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Ministry of  
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# FINAL REPORT

RESEARCH

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20060116

**THE ECOLOGY OF MYCOPLASMA BOVIS STRAINS  
DERIVED FROM CALVES IN WESTERN CANADIAN  
FEEDLOTS**

**Funded by: The Agriculture Development Fund**

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**Prepared by: University of Saskatchewan (U of S)**



## **Final Report**

**The ecology of *Mycoplasma bovis* strains derived from  
calves in western Canadian feedlots**

**(ADF Project #: 20060116)**

**Submitted: March 31, 2010**

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## Summary

This project was co-funded by the Agricultural Development Fund (ADF), the Alberta Beef Producers (ABP), Pfizer Animal Health (Canada), with in-kind support from Pound-Maker Ventures (Lanigan, Saskatchewan). While the original grant application was to cover the commercial feedlot study at Pound-Maker, additional funds from the Alberta Beef Producers and Pfizer Animal Health (Canada) allowed us to expand the scope of the project. Specifically, we conducted a series of smaller projects, each with a specific objective, and each written up in this report as a separate study.

The first of these related studies was a preliminary prevalence study in which we obtained approximately 80 nasopharyngeal (NP) samples from feedlot animals. This study provided data on the prevalence of *Mycoplasma spp.* in healthy and sick cattle, and showed how the prevalence of *Mycoplasma spp.* varied with the number of days the cattle were on feed. The prevalence of *Mycoplasma spp.* varied between feedlots from 20 – 82%. There was a greater chance of isolating *Mycoplasma spp.* from the nasopharyngeal passages of healthy (78%) versus sick animals (44%). And there was a trend towards isolating a greater percentage of *Mycoplasma spp.* from NP samples obtained from calves < 60 days on feed (DOF) (71%) versus calves  $\geq$  60 DOF (44%). The finding that prevalence decreased with the number of days on feed was opposite to what we found in latter studies and this finding may be related to these animals having undergone significant antimicrobial therapy for respiratory disease.

The preliminary study also provided insight into the difficulty of obtaining isolates using transtracheal washes, a procedure that is widely published but which we found was a very poor method of sampling. Isolates from the preliminary study were also used to assist in the development of the polymerase chain reaction (PCR) and random amplified polymorphic DNA (RAPD) molecular assays. Finally, isolates were used to test the viability of storing *Mycoplasma spp.* in a -82°C freezer. From this study we confirmed that *Mycoplasma spp.* isolates maintain their viability in a -82°C freezer for at least 32 months. This study is herein referred to as the **Freezer stability study.**

In addition to the preliminary prevalence study, we ran a study to compare the recovery of *Mycoplasma bovis* from the upper and lower respiratory tract of fall-placed feedlot calves. Nasopharyngeal (NP) swabs were used to sample the upper respiratory tract, while bronchoalveolar lavages (BAL) were used to obtain samples from the cranial lung lobe. Samples were taken from healthy calves at 14 and 90 days on feed (DOF). The prevalence of *Mycoplasma*

*spp.* was compared by sampling site (NP vs BAL) and the number of days on feed (14 and 90). The prevalence of *M. bovis* increased 6 - 8 times over the 76 day study period. At 14 DOF the prevalence of *M. bovis* in the study group was 10% in the upper respiratory tract and 3% in the lungs. By 90 DOF, the prevalence increased to 62% in the upper respiratory tract and 24% in the lungs. Significantly, there was poor agreement in the recovery of *M. bovis* from NP versus BAL at 14 DOF ( $Kappa = 0.18$ ,  $P = 0.06$ ) and no agreement at 90 DOF ( $Kappa = 0.08$ ,  $P = 0.3$ ), which is an important finding because it tells us that colonization of the upper respiratory tract is not indicative of colonization of the lower respiratory tract. *Mycoplasma bovis* isolates were also identified by strain using the AFLP (Amplified Fragment Length Polymorphism) strain differentiation assay. The above study is herein referred to as the **NP-BAL study**.

In addition to the above studies, private practitioners were asked to provide lung tissue samples and joint swabs from confirmed cases of Chronic Pneumonia and Polyarthritis Syndrome (CPPS), the syndrome of fall-placed calves that is associated with *Mycoplasma bovis*. Isolates from these samples were typed by PCR and then subjected to strain differentiation by AFLP testing. Nineteen (19) carcasses, from 10 feedlots, were sampled and the *Mycoplasma bovis* isolates were compared within and between animals. This study is herein referred to as the **Necropsy study**.

While the above studies generated a significant amount of data, the main study involved obtaining NP samples from ~1300 calves in the fall of 2007 and 2008. The NP swabs were taken from each animal at the time of arrival into the feedlot and again at the time of treatment for respiratory disease. The 'on-arrival' and 'treatment' swabs were cultured at the completion of the study for *Mycoplasma spp.* Isolates were then typed by PCR and differentiated into strains by AFLP. This main study is herein referred to as the **Pound-Maker study**.

One of the project's overarching objectives was to distinguish between pathogenic and nonpathogenic strains of *M. bovis* by employing molecular strain differentiation (RAPD and AFLP) to compare *M. bovis* isolates from confirmed CPPS cases to isolates from healthy cattle. Using AFLP, the 'pathogenic' *Mycoplasma bovis* isolates from the necropsy study were differentiated into 3 main clusters or groups (A, B and C). Isolates from the NP-BAL and Pound-Maker studies were all found to cluster within Groups A and B; there were no new clusters from these two sets of samples and none of the samples clustered within Group C. Furthermore, strains from cattle treated for respiratory disease at Pound-Maker tended to cluster in with Group A, while the strains identified in the NP-BAL study clustered mainly with Group B, with some isolates clustering with strain Group A.

It is of interest that McAuliffe *et al* also identified 3 main clusters of *M. bovis* strains that were obtained from cattle in the United Kingdom over a 6 year period. Without doing more intensive genotyping, we cannot say whether these clusters match our clusters.

To review, the original objectives of this project were to: 1) determine the prevalence of *Mycoplasma bovis* in fall-placed feedlot calves; 2) describe the ecology of mycoplasma within pens of feedlot calves; and 3) relate the genetic differences, species and strain variations, to pathogenicity in the field environment. This project showed that the prevalence of *Mycoplasma bovis* varied widely between feedlots, by method of sampling (NP and BAL), and with the length of time the animals were on feed. From these studies we can conclude that *Mycoplasma bovis* is an extremely ubiquitous organism and that it can be readily cultured from the nasopharynx of healthy and sick animals. This project also provided some insight into the ecology of *Mycoplasma spp.* within feedlot pens. The data strain differentiation data shows that similar strains of the organism are spread within pens and within the feedlot. This is supported by the data from the NP-BAL study which correlated an increasing prevalence of *Mycoplasma spp.* with the amount of time in the feedlot. In addition, the calves in the NP-BAL and Pound-Maker tended to be colonized by strains unique to that pen and feedlot. While only 3 unique strains were identified, we found that they clustered by feedlot (study). We also found that animals can be colonized by multiple species of *Mycoplasma*. In the necropsy study, lung and joint isolates from the same animal were typed to two different stains (A and B). With respect to the 3<sup>rd</sup> objective, relating species and strain variations to the pathogenicity, we can conclude that the *Mycoplasma bovis* isolates from healthy and sick cattle can be typed into 1 of 3 clusters (A, B, and C). While all 3 strains were recovered from animals that died or were euthanized because of CPPS, stains A and B were also recovered from healthy cattle. Furthermore, it was also of interest that strain C was never isolated from healthy cattle. Although AFLP was found to be a useful tool for epidemiological studies, allowing researchers to examine strains within an animal, a pen, a feedlot and across feedlots. It was unable to distinguish between pathogenic and nonpathogenic strains of *Mycoplasma bovis*.

# **1. Project overview including objectives, research plan, materials and methods, and amendments to research plan.**

## **1.1 Background**

*Mycoplasma bovis* is a bacterial pathogen associated with several different conditions in cattle including, keratoconjunctivitis, otitis media, mastitis, respiratory disease and arthritis (Alberti *et al.*, 2006; Gagea *et al.*, 2006; Maeda *et al.*, 2003). A lack of cell wall makes *M. bovis* inherently resistant to the  $\beta$ -Lactam family of antibiotics and the bacteria is gaining resistance to most of the other groups of antibiotics commonly used in cattle (Francoz *et al.*, 2005; ter Laak *et al.*, 1993). The surface proteins expressed by *M. bovis* are variable, making an antigenic target for vaccine production difficult and therefore no efficacious vaccine is available for most *M. bovis* associated diseases (Francoz *et al.*, 2005; ter Laak *et al.*, 1993). Chronic Pneumonia and Polyarthritis syndrome (CCPS) is a *M. bovis* associated disease in feedlot cattle that is characterized by persistent respiratory disease despite antibiotic treatment and often subsequent arthritis in one or several joints (Behrens *et al.*, 1996, Le Grand *et al.*, 1996, Lysyansky *et al.*, 1996; 1999; Poumarat *et al.*, 1998; Sachse *et al.*, 2009). The difficulty in treating and preventing this condition makes it of great concern to beef producers as CPPS poses significant welfare and economic concerns in the feedlot (Nicholas and Ayling, 2003).

The prevalence of *M. bovis* has been studied widely in North America, Australia and Europe (Arcongioli, *et al.*, Assie *et al.*, 2009; Ayling *et al.*, 2004; 2007; Bashiruddin *et al.*, 2001; Blackburn *et al.*, 2007; Gadhersohi *et al.*, 1999; Gagea *et al.*, 2006, Le Grand *et al.*, 2002; Wiggins *et al.*, 2007). Results of previously reported prevalence studies vary widely and while this may be due to the different geographical regions there are also discrepancies in the sampling period, sampling method, and species identification method. Also the sampling period appears to affect the prevalence results as several studies have observed an increase in *M. bovis* isolation with an increase in the number of days the animals have been in the feedlot (Allen *et al.*, 1992; Rosendal and Martin *et al.*, 1986). The variation in results due to sampling differences and methodology among reported prevalence studies from other regions makes it difficult to make any inferences about the prevalence of *Mycoplasma* in western Canada.

While *M. bovis* has been shown to cause CPPS in both gnotobiotic and conventional calves, it is also known to colonize the respiratory tracts of seemingly healthy cattle (Allen *et al.*, 1991; 1992; Hirose *et al.*, 2003; Rosendal and Martin, 1986; Sachse *et al.*, 2009; ter Laak *et al.*, 1992b; Thomas *et al.*, 2002; Wiggins *et al.*, 2007). This would suggest that there is variability of

pathogenicity within the species. Molecular strain differentiation has recently been utilized to try and identify different strains in *M. bovis* epidemiology studies. Random Amplified Polymorphic DNA (RAPD) is a strain differentiation method that utilizes a random nucleotide primer in standard PCR reactions in order to reveal polymorphisms between the genomes of strains (Butler *et al*, 2002; McAuliffe *et al*, 2004). The method has been utilized in *M. bovis* epidemiology studies and has demonstrated high discriminatory power when compared to other methods (Butler *et al*, 2002; McAuliffe *et al*, 2004). A recent study conducted to compare strain differentiation methods found that RAPD was able to separate 56 *M. bovis* isolates collected over a 6 yr period in the United Kingdom into two genetically distinct clusters (McAuliffe *et al*, 2004). The same study also compared a strain differentiation method known as Amplified Fragment Length Polymorphism (AFLP) that utilizes a double digest with restriction enzymes followed by a PCR of the resultant fragments in order to identify genomic polymorphisms within a species (Kokotovic *et al*, 1999; McAuliffe *et al*, 2004). The study found that this method was consistent with RAPD and produced the same genetically distinct clusters, however AFLP was able to identify two sub-groups within one of these clusters and therefore may have higher discriminatory power than RAPD (McAuliffe *et al*, 2004). While these strain differentiation methods do appear to be suitable for *M. bovis* and have provided useful information regarding *M. bovis* epidemiology.

## **1.2 Project Objectives**

The objectives of this study were to: 1) determine the prevalence of *Mycoplasma bovis* in fall-placed feedlot calves; 2) describe the ecology of mycoplasma within pens of feedlot calves; and 3) relate the genetic differences, species and strain variations, to pathogenicity in the field environment.

## **1.3 Research Plan**

### ***Animal sampling procedure***

A copy of the research plan, as it was presented in the original grant application, is provided in Appendix A. Briefly, the plan was to obtain deep nasopharyngeal (nasal) swabs from ~2,400 feedlot calves. These calves were 'fall-placed' calves, meaning that they would be weaned shortly before entering the feedlot. Typically, commercial feedlots divide their feedlot into pens of ~300 animals. The plan was to sample 4 pens of calves (~1,200) in the fall of 2007 and a similar number of calves in the fall of 2008. The reason for sampling in the fall is because

the organism of interest, *Mycoplasma bovis*, manifests itself as *chronic pneumonia and polyarthritis syndrome* (CPPS), which is restricted to fall-placed calves. Therefore, the window of opportunity for studying the disease is relatively limited (October-December). Pound-Maker feedyards (Lanigan Saskatchewan) was identified as the feedlot from which all the samples would be obtained.

The study protocol stipulated that a single nasopharyngeal (NP) swab be obtained from each calf within 7 days of entry into the feedlot. If any of the calves were treated for respiratory disease, then a second NP sample was taken at the time of treatment. This procedure was repeated for each subsequent treatment. Lastly, if the calf died of confirmed CPPS, then a sample of lung tissue was also retained. By collecting these samples, we hoped to determine whether the stains of *Mycoplasma bovis* resident in the nasal passages of calves upon entry into the feedlot were the same strains as those found at time of first treatment and in calves diagnosed as having died of CPPS. These data would provide insight into whether certain strains are more pathogenic than others.

### ***Microbiological culturing and PCR species identification procedures***

#### **Initial culturing procedure**

All the samples were initially processed by Dr. Manuel Chirino, at the Western College of Veterinary Medicine. The samples were thawed and streaked onto Hayflick's agar and incubated at 37°C in 5% CO<sub>2</sub> for 48hrs. Following incubation, 4 presumptive *Mycoplasma spp.* colonies were selected and sub-cultured onto Hayflick's agar under the same conditions for an additional 48 hrs at 37°C in 5% CO<sub>2</sub>. These subcultured colonies were then inoculated into 4-10 mL of Hayflick's broth under the same temperature and atmospheric conditions for 3-7 days. Stocks of 0.50 - 0.75 mL Hayflick's broth and 0.50 - 0.75 mL litmus milk were saved in 1.5 mL Eppendorf tubes at -82 °C.

#### **PCR assay**

The presumptive *Mycoplasma spp.* broth cultures were then divided, one retained by Dr. Chirino and the second sent to Dr. Andrew van Kessel's laboratory, where his graduate student, Rose Whelan, performed the polymerase chain reaction (PCR) assay to identify the species of *Mycoplasma*. Samples that were positively identified by PCR as *Mycoplasma bovis* became candidates for strain testing.

The following is a description of how the PCR assay was performed. Fresh broth cultures (4-10mL) were centrifuged at 3000 rpm for 1hr and the supernatant removed. Genomic DNA was extracted from the resultant bacterial pellet using Purelink<sup>TM</sup> Genomic DNA Kit (Invitrogen, Carlsbad, CA) according to the instruction manual for gram negative bacterial cell lysates. The extracted DNA was quantified and examined for purity using UV absorbance (NanoDrop® ND-1000 spectrophotometer; NanoDrop Technologies, Inc., Wilmington, USA) and stored at -80°C until use.

Polymerase Chain Reactions (PCR) was employed to amplify the 16S rRNA gene for nucleotide sequencing. The PCR reaction mixture included 1x PCR Buffer (Invitrogen, Burlington, ON), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 3 mM BSA, 0.5 mM universal 16S rRNA gene forward primer (5'-GAGTTTGATCCTGGCTCAG-3'), 0.5 mM universal 16S rRNA gene reverse primer (5'-GWATTACCGCGGCKGCTG-3'), at least 10ng of Purelink<sup>TM</sup> extracted genomic DNA, 0.5ul Taq polymerase (Invitrogen, Burlington, ON) and ddH<sub>2</sub>O to bring the reaction mixture volume to 50µl. A hot start at 95°C for 10 min in an AmpliTron II thermocycler (ThermoFisher, Barnstead International, Dubuque, Iowa) was utilized before Taq was added to the reaction mixture. PCR amplification continued for 40 cycles using a denaturing phase of 95°C for 30sec, an annealing phase at 50°C for 30sec, and an extension phase at 72°C for 30sec.

Successful PCR amplification was confirmed by electrophoresis of 50 µL of the completed PCR reaction with 10 µL of a 0.5% bromophenol loading buffer using a 1.5% agarose gel containing 0.5µg/mL ethidium bromide at 100 V for 1.5 hours (BioRad Model 1000/500 Power Supply, BioRad, Hercules, California). PCR products of expected size were extracted from the agarose gel with the Qiaex II Gel Extraction Kit as outlined in the DNA Extraction from Agarose Gels section of the Qiaex II handbook (Qiagen, Mississauga, Ontario). DNA in gel-purified PCR products was quantified with the NanoDrop® ND-1000 spectrophotometer and submitted to the National Research Council, Plant Biotechnology Institute (Saskatoon, SK) for DNA sequencing.

Raw nucleotide sequence data was processed with the PREGAP v1.5 and GAP4 v4.10 programs of the Staden package (Dear and Staden 1991) in order to align forward and reverse sequences, confirm sequence quality and delete primer sequences. Trimmed, quality sequences were uploaded to the 16S Ribosomal Database Project II (Cole *et al.*, 2207; Cole *et al.*, 2009) website and the Sequence Match tool used to identify the nearest bacterial species match. Phylogenetic analysis and tree construction of sequences was performed using ARB phylogenetic software (Ludwig *et al.*, 2004).

## **RAPD and AFLP strain differentiation assays**

Two different strain differentiation tests were developed, RAPD and AFLP.

### **Random amplified polymorphic DNA (RAPD) Strain Differentiation**

A 50 $\mu$ L RAPD reaction was prepared containing 1x PCR Buffer (Invitrogen, Burlington, ON), 1.5mM MgCl<sub>2</sub>, 0.2mM dNTPs, 0.1% Triton X-100, 1mM primer Hum4 5'-ACGGTACAT-3' (Hotzel *et al.*, 1998), at least 50ng of sample DNA, 5ul Taq polymerase (Invitrogen, Burlington, ON) and UV ddH<sub>2</sub>O to bring the reaction mixture volume to 50 $\mu$ L. The RAPD reaction mixtures were subjected to 95°C for 3 min and then 40 cycles of 95°C for 30sec, 37°C for 30sec, and 72°C for 30sec in the AmpliTron II thermocycler (Thermolyne, Barnstead International, Dubuque, Iowa). RAPD amplification products were then assessed with electrophoresis on a 2% agarose gel containing 0.5 $\mu$ g/mL ethidium bromide. The voltage used was initially 140V for 5min followed by 90V for 1.5hr (BioRad Model 1000/500 Power Supply, BioRad, Hercules, California). Gels were visualized under UV illumination.

### **Amplified Fragment Length Polymorphism (AFLP) Strain Differentiation**

Extracted genomic DNA was amplified with the Illustra GenomiPhi HY DNA Amplification Kit as described in the manual (GE Healthcare, Buckinghamshire, UK). Approximately 150-500ng of DNA was digested with 10U of MfeI and 10U of BglII restriction enzymes (New England Biolabs, Beverly, Mass.) in a buffer previously described by Kokotovic *et al* (1999) at 37°C for 6 hrs. Double stranded adapters were prepared as described in Kokotovic *et al* (1999). A ligation reaction consisting of 5ul of restriction enzyme digest, 10pmol of a double stranded BglII adapter, 100pmol of a double stranded MfeI adapter, 1U of T4 DNA ligase (New England Biolabs, Beverly, Mass.) and the previously described buffer (Kokotovic *et al*, 1999) was incubated at room temperature for 16-20hrs.

PCR amplification from 1 $\mu$ L of a 1:10 diluted ligation reaction product was performed. PCR reactions consisted of a 1x PCR Buffer (Invitrogen, Burlington, ON), 2.5mM MgCl<sub>2</sub>, 0.2mM of each of the four dNTPs, 65ng of MFE1-0 primer (Kokotovic *et al*, 1999), 65ng of BGL2F-0 primer (Kokotovic *et al*, 1999) 0.3ul Taq polymerase (Invitrogen, Burlington, ON) and UV ddH<sub>2</sub>O to bring the reaction mixture volume to 50 $\mu$ L. Amplification was run in a Biorad C1000TM Thermal Cycler (Biorad, Mississauga, ON). An initial 3min denaturation at 95°C was performed, followed by 40 cycles of denaturation at 95°C for 60s, annealing at 54°C for 60s and elongation at 72°C for 90s. Amplification completed with a final elongation step of 72°C for 10min.

Amplification fragments were analyzed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster City, CA). Mixtures of 1uL of 1:50 (v/v) dilution in ddH<sub>2</sub>O of amplification product, 1uL Genescan 600 Liz internal size standard (Applied Biosystems, Foster City, CA) and 8uL formamide were denatured at 95°C for 5 min and placed on ice for 2 min before detection.

Densitometric curves from the ABI 3130x Genetic Analyzer were uploaded into Genemapper v3.7 and 5 bin analysis for AFLP, in which the software searches for significant peaks every 5 nucleotides, was performed. Compared across all samples, restriction fragments within a range of 5 bp were considered a single peak and peak areas combined under a single size (bp) designation. Resulting peak area profiles were imported into Bionumerics v5.1 (Applied Maths, Austin, TX). Clusters were made based on Dice similarity coefficient similarity using the unweighted pair group method with arithmetic mean algorithm with optimization and position tolerance settings at 0% and 1% respectively. A reproducibility trial was conducted in which nine samples, at least two from each AFLP plate analyzed, were run and analyzed in duplicate with the identical AFLP protocol, supplies, equipment and software.

#### **Establishment of *M. bovis* strain differentiation methodology**

The RAPD strain methodology adapted from McAuliffe *et al* (2004) failed to produce reliable and reproducible results. Success of the RAPD protocol, as determined by presence of bands when visualized with ethidium bromid under UV light, was inconsistent between duplicate runs. This related to both an inconsistent pattern in the number and intensity of the bands. Unfortunately, almost a year was devoted to this assay; multiple DNA purification kits, along with different reagents, and different reaction parameters, were tried before concluding that this assay was unsuitable for this study.

The AFLP protocol resulted in much higher reliability and reproducibility of results. Only minor changes in densitometric curve and band assignment during analysis between the nine duplicate runs resulted was observed and therefore similarity score, based on the dice coefficient, between duplicate samples was consistently above 92% (Appendix B). Fingerprints consisting of between 20-60 fragments were produced for single samples with the AFLP protocol.

#### **1.4 Amendments to the Research Plan**

There were a number of minor and major amendments to the proposed Research Plan. Because CPPS is primarily a disease of fall-placed calves, there was a limited window of opportunity (October – December) for sampling the calves. At the time of submission of the funding application, we considered sampling 600 calves in the fall of 2006, before funding was

granted. However, logistics prevented this from happening, thus the first calves were sampled at Pound-Maker in the fall of 2007. Unfortunately, this was a very unusual year for the cattle markets and an unusually high number of Canadian calves were exported to the United States. As a result, many of the large feedlots did not purchase fall-placed calves, or they delayed their purchases until very late in the fall when prices had moderated. As a result, we were only able to sample two pens of cattle (Lots 681 and 142) in October at Poundmaker, comprising approximately 600 calves. Pound-Maker did purchase more calves later on in November but they entered the feedlot over a very short time span and the feedlot processing crew could not accommodate our research.

In addition to recruiting only half the desired number of calves, the incidence of respiratory disease, and particularly CPPS, was very low in 2007. This may have been related to the weather, lower density of fall-placed calves, or the introduction of a new long-acting antimicrobial (Draxxin™) developed specifically for respiratory disease in cattle. Complicating this lack of cases, the feedlot crew, who was to obtain the nasal swabs at the time of treatment, did not always remember to sample the sick cattle and they overlooked sampling the healthy animals as matched controls.

Heading into the fall of 2008 we were faced with trying to recruit 1,800 animals onto the study at Pound-Maker, which would have provided the desired study number of 2,400. However, because this feedlot is first and foremost a commercial operation, we had to accept that swabbing sick animals may get overlooked by the processing crews. And because the feedyard is 90 minutes from Saskatoon, it would have been impractical to travel that distance daily to collect the swabs. Therefore, we elected to run the 2<sup>nd</sup> half of the study in the fall of 2008 at the University of Saskatchewan feedlot, only minutes away. However, the U of S feedlot uses much smaller pens, which decreases the incidence of respiratory disease but given the concerns we encountered the previous year, we elected to sample calves at the U of S feedlot in 2008. As will be discussed, the incidence of respiratory disease in 2008 at the U of S feedlot was exceptionally low and hence only the samples from the 2007 Pound-Maker feedlot study were analyzed.

As discussed, we also encountered significant issues with the RAPD assay. A previous published report by McAuliffe *et al* found that the RAPD and AFLP results were comparable and hence we elected to use the RADP assay. However, after nearly a year in development we were forced to abandon this assay and switch to the AFLP. This became an issue because we had run out of both time and funding to run this assay on all the *M. bovis* isolates.

## **2. Preliminary *Mycoplasma* spp. prevalence testing**

### **2.1 Study Objectives**

The objective of this preliminary study was fourfold: 1) obtain preliminary data on the prevalence of *Mycoplasma bovis* in various ages of beef cattle on feed; 2) compare the recovery of *Mycoplasma* spp. from the nasopharyngeal swabbing technique versus transtracheal washes; 3) provide *M. bovis* isolates for use in developing molecular typing techniques, specifically, random amplified polymorphic DNA (RAPD) analysis; and 4) determine the viability of *Mycoplasma* spp. isolates after storage in either liquid nitrogen (N<sub>2</sub>) or -82°C freezers.

### **2.2 Materials and Methods**

Samples were obtained from five different feedlots, 2 from Saskatchewan and 3 from Alberta. Depending on the feedlot, nasopharyngeal (NP) samples and transtracheal washes (TTW) were obtained; not all locations were set-up for TTW sampling. The methods for collecting the NP and TTW samples are described in Appendix C.

The cattle included a mix of sick and healthy animals, and the number of days on feed (DOF) ranged from 14 to > 90d.

During the initial phase of this project a number of different sampling techniques and materials were tested. Initially, animals were swabbed using commercially available swabs (Becton-Dickenson). While these samples worked on smaller calves and were sufficient for a mid-nasal cavity sample, they were too short for "deep" nasopharyngeal swabbing. In addition, these swabs did not have any special storage media, rather they were a sterile cotton tip. These shorter Becton-Dickenson (BBL™ culture swab) swabs were then replaced by Tieglund swabs, which were 60 cm long and designed to sample the reproductive tract (uterus) of mares. These swabs were able to reach to the back of the nasal passages of mature animals. After trying a number of different suppliers, Reproductive Resources was chosen because they could provide 60 cm double-guarded swabs, along with Ames transport media.

Duplicate, and in some cases triplicate, nasopharyngeal (NP) swabs were taken from each animal, alternating between nostrils. Triplicate samples were taken from 11 animals at the U of S feedlot along with 9 transtracheal washes (Table 2.1). Twenty (20) animals were sampled from the feedlot "H". Six of the 20 samples were collected in triplicate and the remainder in duplicate. Duplicate NP were obtained at the "H", "P" and "C" feedlots; 4 TTW were also done at the "C"

feedlot but all cultures were negative for *Mycoplasma spp.* Appendix D provides a full accounting of the number of samples taken per farm and their respective culture results.

In total, there were 12 triplicate samples (7 U of S, 5 "W" Feedlot). Triplicate samples obtained from the U of S feedlot and W feedlot were divided with 2 swabs going into a -82°C freezer and 1 swab placed in liquid nitrogen. All other samples were obtained in duplicate and stored in a -82°C freezer. Some of the swabs placed in liquid nitrogen were destroyed because they were not manufactured for these extreme cold conditions.

Table 2.1 Number of samples by date, sampling method, and location (feedlot).

| Feedlot <sup>a</sup> | Date of sampling | Positive NP samples | Positive TTW |
|----------------------|------------------|---------------------|--------------|
| U of S               | December 2006    | 7 (triplicate)      | 6            |
| W                    | March 2007       | 5 (triplicate)      | 0            |
| H                    | March 2007       | 5 (duplicate)       | 0            |
| P                    | March 2007       | 8 (duplicate)       | 0            |
| C                    | March 2007       | 8 (duplicate)       | 0            |

<sup>a</sup>To protect the privacy of the cooperating feedlots, only the first letter of their name has been used to identify the feedlot. U of S represents the University of Saskatchewan's feedlot.

## 2.3 Results

Thirty-five (35) swabs were positive for *Mycoplasma spp.* and the prevalence of *Mycoplasma spp.* from all the NP samples varied between feedlots from 20 – 82%. There was a greater chance of isolating *Mycoplasma spp.* from the nasopharyngeal passages of healthy (14/18 or 78%) versus sick animals (21/48 or 44%) (OR = 1.5; 1.2-15.7;  $P = 0.03$ ). And there was a trend towards isolating a greater percentage of *Mycoplasma spp.* from NP samples obtained from calves < 60 days on feed (DOF) (15/21; 71%) versus calves ≥ 60 DOF (20/45; 44%) (OR = 1.13, CI 1.0 – 9.5;  $P = 0.07$ ).

Table 2.2 is a summary of the *Mycoplasma spp.* isolates from the 5 feedlots:

- University of Saskatchewan cattle. Samples were obtained from healthy cattle ~ 60 DOF. Nine of eleven NP samples (82%) and 6/9 (67%) TTW samples were positive for *M. spp.* However, 2 of the NP samples had very low recoveries with the microbiologist (Dr. Manuel Chirino-Trejo) reporting "few colonies" isolated.
- The second feedlot, "W", was located in southeast Saskatchewan. Twenty animals were sampled and all came from the 'sick pen', hence they had been given antimicrobial

therapy prior to sampling. Of the 20 animals, 15 were > 150 DOF, while 5 were classified as new arrivals (<30 DOF). The rates of recovery of *Mycoplasma spp.* were very low for both healthy 3/15 (20%) and sick animals 1/5 (20%). No TTW were obtained from any of the animals.

- Ten NP samples were taken at the “H” feedlot. All samples were obtained from animals in the sick pen and all were >90 DOF. The prevalence of *M. spp.* was 50% (5/10). Both NP and TTW samples were obtained from the “P” feedlot. All samples were obtained from animals in the sick pen and all were >90 DOF. Eight of 15 NP (53%) were positive for *M. spp.*, while none (0/4) of the TTW samples were positive for *M. spp.*
- Ten NP samples were obtained from the “C” feedlot. *Mycoplasma spp.* were isolated from 2 healthy calves < 14 DOF. Similarly, *Mycoplasma spp.* were isolated from 3/3 pen mates that were sick and from 3/5 healthy animals that were ~150 DOF. Overall, the prevalence of *Mycoplasma spp.* was 80% (8/10).

Table 2.2 Breakdown of the prevalence of *Mycoplasma spp.* isolates from healthy and sick cattle from 5 different feedlots.

| Feedlot | Days on Feed | Health Status | Nasopharyngeal | Transtracheal Wash <sup>+</sup> |
|---------|--------------|---------------|----------------|---------------------------------|
| U of S  | ~ 60         | Healthy       | 9/11           | 6/9                             |
| W       | >150         | Sick          | 4/15           |                                 |
|         | < 30         | Sick          | 1/5            |                                 |
| H       | > 90         | Sick          | 5/10           |                                 |
| P       | > 90         | Sick          | 8/15           | 0/4                             |
| C       | <14          | Healthy       | 2/2            |                                 |
|         | < 14         | Sick          | 3/3            |                                 |
|         | ~150         | Healthy       | 3/5            |                                 |

<sup>+</sup> TTW samples were only obtained at the U of S and “P” feedlots.

## 2.4 Conclusions

The preliminary study met the 4 original objectives. Firstly, it provided data on the prevalence of *Mycoplasma spp.*, which was a critical piece of information for doing power calculations for subsequent studies. Secondly, although the procedure for performing TTW in cattle has been published in the literature, performing this technique was very unrewarding. The procedure is relatively easy and innocuous, but as later confirmed with the bronchoscope, the

bovine has a very vertically oriented trachea and hence the lavage fluid tends to quickly gravitate to the lungs, making it very difficult to aspirate a sample for culture. This funding necessitated that we obtain future samples using the bronchoalveolar lavage (BAL) technique, which was much more invasive and time-consuming. Thirdly, these preliminary cultures provided genetic material for Rose Whelan to develop her polymerase chain reaction (PCR) assay for speciating the *Mycoplasma spp.* The fourth objective, freezer stability study, is discussed as a separate study in the next section.

### 3. Freezer stability study

#### 3.1 Objective

The objective of this study was to assess the viability of *Mycoplasma spp.* when stored at -82°C for 3 months, 6 months and 32 months.

This study was conducted in the advance of the larger 'feedlot' studies to determine whether the *Mycoplasma spp.* would remain viable under long-term storage. Published literature indicated that that *Mycoplasma spp.* are very hardy and would survive long-term storage. However, Dr. Chirino noted that in many cases the storage conditions were in liquid nitrogen (N<sub>2</sub>), whereas because of cost and ease of storage, the samples collected in this project would be stored in -82°C freezers.

#### 3.2 Materials and Methods

As discussed previously, preliminary sampling was conducted on calves from the University of Saskatchewan (U of S) feedlot and from a number of privately-owned feedlots in Saskatchewan ("W") and Alberta ("H", "P", and "C"). The stability study spanned 3 time frames (3 months, 6 months and 32 months).

Triplicate samples were obtained from the 11 calves at the U of S and 6 calves from the feedlot "W". Two of the swab samples were stored at -82°C and a third in liquid nitrogen. Unfortunately, these commercial swabs were not made for storage in liquid N<sub>2</sub> and hence only 3 duplicate sets of samples were salvaged from the liquid N<sub>2</sub> canister.

Samples from the U of S were used in the 3 and 6 month stability studies, while samples from the W, H, P and C feedlots were used in the 32 month stability study, see Table 3.1.

Table 3.1 Number of samples by feedlot, date of sample collection, and date(s) of 1<sup>st</sup> culture.

| Feedlot | Date of sampling | 1 <sup>st</sup> culture | 2 <sup>nd</sup> and 3 <sup>rd</sup> cultures | Study         |
|---------|------------------|-------------------------|----------------------------------------------|---------------|
| U of S  | December 2006    | Feb 2007                | May and Aug. 2007                            | 3 and 6 month |
| W       | March 2007       | May 2007                | November 2009                                | 32 month      |
| H       | March 2007       | May 2007                | November 2009                                | 32 month      |
| P       | March 2007       | May 2007                | November 2009                                | 32 month      |
| C       | March 2007       | May 2007                | November 2009                                | 32 month      |

### 3.3 Results

Tables 3.2 and 3.3 compare the culture results from the initial culture (1<sup>st</sup> Culture) and 2<sup>nd</sup> culture dates for the 3 and 6 month stability studies, respectively. Both studies used a combination of NP and TTW samples.

Table 3.2 Short-term (3 month) stability study using swabs and transtracheal washes stored in -82°C freezers and liquid N<sub>2</sub>.

| Feedlot                                                                      | Sample ID | Storage            | 1 <sup>st</sup> Culture | Results | 2 <sup>nd</sup> Culture | Results |
|------------------------------------------------------------------------------|-----------|--------------------|-------------------------|---------|-------------------------|---------|
| Nasopharyngeal swabs stored in -82°C freezer; samples obtained December 2006 |           |                    |                         |         |                         |         |
| U of S                                                                       | 001       | -82°C              | Feb 2007                | 2+      | May 2007                | 2+      |
|                                                                              | 002       | -82°C              | Feb 2007                | Few     | May 2007                | Neg     |
|                                                                              | 003       | -82°C              | Feb 2007                | Few     | May 2007                | Neg     |
|                                                                              | 004       | -82°C              | Feb 2007                | 2+      | May 2007                | 2+      |
|                                                                              | 005       | -82°C              | Feb 2007                | 1+      | May 2007                | 1+      |
|                                                                              | 006       | -82°C              | Feb 2007                | Neg     | May 2007                | Neg     |
|                                                                              | 007       | -82°C              | Feb 2007                | Neg     | May 2007                | Neg     |
|                                                                              | 008       | -82°C              | Feb 2007                | 1+      | May 2007                | 1+      |
|                                                                              | 009       | -82°C              | Feb 2007                | 2+      | May 2007                | 2+      |
|                                                                              | 010       | -82°C              | Feb 2007                | 1+      | May 2007                | 1+      |
|                                                                              | 011       | -82°C              | Feb 2007                | 2+      | May 2007                | 2+      |
| Transtracheal washes (TTW) stored in -82°C freezer and liquid N <sub>2</sub> |           |                    |                         |         |                         |         |
|                                                                              | 001       | TTW                | Feb 2007                | N/S     | May 2007                | N/S     |
|                                                                              | 002       | -82°C              | Feb 2007                | N/S     | May 2007                | N/S     |
|                                                                              | 003       | -82°C              | Feb 2007                | 1+      | May 2007                | 1+      |
|                                                                              | 004       | Liq N <sub>2</sub> | Feb 2007                | 1+      | May 2007                | 1+      |
|                                                                              | 005       | -82°C              | Feb 2007                | Neg     | May 2007                | Neg     |
|                                                                              | 006       | -82°C              | Feb 2007                | Few     | May 2007                | Few     |
|                                                                              | 007       | Liq N <sub>2</sub> | Feb 2007                | 1+      | May 2007                | 1+      |
|                                                                              | 008       | Liq N <sub>2</sub> | Feb 2007                | 1+      | May 2007                | 1+      |
|                                                                              | 009       | -82°C              | Feb 2007                | 2+      | May 2007                | 2+      |
|                                                                              | 010       | -82°C              | Feb 2007                | Neg     | May 2007                | Neg     |
|                                                                              | 011       | -82°C              | Feb 2007                | Neg     | May 2007                | Neg     |

Table 3.3 Medium-term (6 month) stability study using swabs and transtracheal washes stored in -82°C freezers.

| Feedlot | Sample ID | Sample | 1 <sup>st</sup> culture | Results | 2 <sup>nd</sup> culture | Results |
|---------|-----------|--------|-------------------------|---------|-------------------------|---------|
| U of S  | 001       | NP     | Feb 2007                | 2+      | Aug 2007                | 1+      |
|         | 003       | NP     | Feb 2007                | "few"   | Aug 2007                | "few"   |
|         | 004       | NP     | Feb 2007                | 2+      | Aug 2007                | 2+      |
|         | 006       | NP     | Feb 2007                | "few"   | Aug 2007                | No      |
|         | 009       | NP     | Feb 2007                | 2+      | Aug 2007                | 3+      |
|         | 004       | TTW    | Feb 2007                | 1+      | Aug 2007                | Pos     |
|         | 007       | TTW    | Feb 2007                | No      | Aug 2007                | No      |
|         | 008       | TTW    | Feb 2007                | 1+      | Aug 2007                | Pos     |

Nasopharyngeal samples obtained in March 2007 from 4 different feedlots were collected using a double-guarded 60 cm swab. The swab was then placed in Ames transfer media and stored in a -82°C freezer for 32 months. Table 3.4 shows the results of 1<sup>st</sup> and 2<sup>nd</sup> cultures.

Table 3.4 Long-term (29 month) stability study of NP swabs stored in -82°C freezer.

| Feedlot | Sample ID | Sample Date | Sample | 1 <sup>st</sup> culture | Results | 2 <sup>nd</sup> culture | Results |
|---------|-----------|-------------|--------|-------------------------|---------|-------------------------|---------|
| W       | 002       | Mar 07      | NP     | May 2007                | Neg     | Aug 2009                | Neg     |
|         | 004       | Mar 07      | NP     |                         | Pos     |                         | Neg     |
|         | 006       | Mar 07      | NP     |                         | Neg     |                         | Neg     |
|         | 007       | Mar 07      | NP     |                         | Pos     |                         | Pos     |
|         | 008       | Mar 07      | NP     |                         | Neg     |                         | Neg     |
|         | 010       | Mar 07      | NP     |                         | Pos     |                         | Pos     |
|         | 012       | Mar 07      | NP     |                         | Neg     |                         | Pos     |
|         | 014       | Mar 07      | NP     |                         | Pos     |                         | Pos     |
|         | 016       | Mar 07      | NP     |                         | Pos     |                         | Pos     |
|         | 020       | Mar 07      | NP     |                         | Neg     |                         | Neg     |
| H       | 001       | Mar 07      | NP     | May 2007                | Neg     | Aug 2009                | Neg     |
|         | 004       | Mar 07      | NP     |                         | Neg     |                         | Neg     |
|         | 006       | Mar 07      | NP     |                         | Pos     |                         | Pos     |
|         | 007       | Mar 07      | NP     |                         | Pos     |                         | Pos     |

|   |      |        |     |          |     |           |          |
|---|------|--------|-----|----------|-----|-----------|----------|
| P | 001  | Mar 07 | NP  | May 2007 | Neg | Aug 2009  | Pos      |
|   | 002  | Mar 07 | NP  |          | Pos |           | Pos      |
|   | 004  | Mar 07 | NP  |          | Neg |           | Neg      |
|   | 007  | Mar 07 | NP  |          | Pos |           | Pos      |
| C | 001  | Mar 07 | NP  | May 2007 | Pos | Aug 2009  | Neg      |
|   | 002  | Mar 07 | NP  |          | Pos |           | Pos      |
|   | 003  | Mar 07 | NP  |          | Pos |           | Pos      |
|   | 004  | Mar 07 | NP  |          | Pos |           | Pos      |
|   | 005  | Mar 07 | NP  |          | Pos |           | Pos      |
|   | 006  | Mar 07 | NP  |          | Pos |           | Pos      |
|   | 007  | Mar 07 | NP  |          | Pos |           | Pos      |
|   | 008  | Mar 07 | NP  |          | Neg |           | Pos      |
|   | 009  | Mar 07 | NP  |          | Neg |           | Pos      |
|   | 010  | Mar 07 | NP  |          | Pos |           | Pos      |
| W | W001 | Mar 07 | BBL | May 2007 | Neg | August 13 | Positive |
|   | W003 | Mar 07 | BBL |          | Neg |           | Positive |
|   | W004 | Mar 07 | BBL |          | Pos |           | No       |
|   | W006 | Mar 07 | BBL |          | Neg |           | Positive |
|   | W015 | Mar 07 | BBL |          | Neg |           | No       |
|   | W016 | Mar 07 | BBL |          | Pos |           | No       |
|   | W017 | Mar 07 | BBL |          | Neg |           | No       |

<sup>1</sup> The microbiological culture results for the May 2007 culture were reported as either "Mycoplasma spp. isolated" or "No Mycoplasma spp. isolated", that is, "Positive" or "Negative".

### 3.4 Conclusions

In general, there was good agreement between the 1<sup>st</sup> and 2<sup>nd</sup> culture results for the 3, 6 and 32 months studies. These data showed that *Mycoplasma bovis* isolates remain viable when stored in a -82°C for up to 32 months. Therefore, we had confidence in knowing that storing the samples in a freezer until the completion of the study would not alter the culture results.

## **4. Preliminary comparison testing of nasopharyngeal (NP) and bronchoalveolar lavage (BAL) techniques.**

### **4.1 Objective**

The objective of this study was to compare the prevalence of *Mycoplasma spp.* recovered from the nasal cavity using nasopharyngeal (NP) swabs to those obtained from the lung field by bronchoalveolar lavage (BAL). This study was done as a precursor to a large NP-BAL study, which is described in Section 5.

### **4.2 Materials and Methods**

#### ***BAL sampling procedure***

A series of experiments were conducted involving cattle at the University of Saskatchewan feedlot. The BAL technique involved advancing a 90 cm optical endoscope (Olympus; Markham, Ontario, Canada) through the left nostril to the accessory lung lobe. Once 'seated' in the opening of the bronchi, a premeasured length of sterile polypropylene catheter material was inserted into the scope and passed to the rostral end of the fiberoptic scope. While visualizing the catheter through the scope, the catheter was advanced another 5-10 cm into the main bronchi of the accessory lung lobe. Approximately 60 mL of physiological saline was then flushed via the catheter into the accessory lung lobe and within 10 seconds it was aspirated back into the syringe. In some instances, the saline gravitated into the lung field and no aspirate was collected, in which case the lung was lavaged with a 2<sup>nd</sup> 60 mL of saline. Typically 1-5 mL was aspirated and a small aliquot (0.5 – 1.0 mL) was placed in 1.2 mL cryogenic vial (Nalgene™). In some instances, duplicate samples were obtained.

#### ***Sampling of animals***

In November 2007, a duplicate set of NP and BAL samples were obtained from 10 calves from the U of S feedlot (Group 1). One set of samples was submitted directly as fresh (unfrozen) samples to Dr. Chirino's laboratory for culturing, while the second set was retained in a -82°C freezer. An additional 4 calves (Group 2) from the U of S feedlot were sampled within a week of the 1<sup>st</sup> group (November, 2007).

### 4.3 Results

Table 4.1 shows the culture results from the NP and BAL samples obtained from Groups 1 and 2. Culture results for Group 1 were similar, regardless of the method of sampling (NP or BAL). In 3/10 calves in Group 1 no *Mycoplasma spp.* could be cultured from the NP samples, and the BAL samples for these same 3 calves were also negative. Lack of *Mycoplasma spp.* isolates from the BAL technique was more common in Group 2, where *Mycoplasma spp.* were obtained from 4/5 NP samples and 1/5 BAL samples.

Table 4.1 NP and BAL sample culture results.

| Group   | Sample type | ID    | Result       |
|---------|-------------|-------|--------------|
| Group 1 | NP          | N-001 | 1+           |
|         |             | N-002 | 1+           |
|         |             | N-003 | 1+           |
|         |             | N-004 | Few colonies |
|         |             | N-005 | Neg          |
|         |             | N-006 | 1+           |
|         |             | N-007 | Neg          |
|         |             | N-008 | 1+           |
|         |             | N-009 | 1+           |
|         |             | N-010 | Neg          |
|         | BAL         | N-001 | Neg          |
|         |             | N-002 | Few colonies |
|         |             | N-003 | 1+           |
|         |             | N-004 | 1+           |
|         |             | N-005 | Neg          |
|         |             | N-006 | 1+           |
|         |             | N-007 | 1+           |
|         |             | N-008 | Neg          |
|         |             | N-009 | 1+           |
|         |             | N-010 | Neg          |
| Group 2 | NP          | #61   | 2+           |
|         |             | #62   | 1+           |
|         |             | #64   | Neg          |
|         |             | #86   | Few colonies |
|         |             |       |              |

|  |     |     |              |
|--|-----|-----|--------------|
|  |     | #88 | Few colonies |
|  | BAL | #61 | Few          |
|  |     | #62 | Neg          |
|  |     | #64 | Neg          |
|  |     | #86 | Neg          |
|  |     | #88 | Neg          |

#### 4.4 Conclusions

While the number of calves sampled was relatively low, this preliminary study allowed us to develop skills for conducting the BAL sampling procedure. The culture results also provided some data on the prevalence of *Mycoplasma spp.* isolates from the NP and BAL samples. Furthermore, these results allowed us to examine, at the level of the individual animal, the level of agreement between the NP and BAL samples. In only 1 instance the NP was negative and the BAL positive, whereas in 4 instances the NP was positive and the BAL negative. Overall, in 10/15 calves, the NP and BAL were in agreement (either both positive or negative).

## **5. Prevalence of *M. bovis* in healthy feedlot steers using nasopharyngeal (NP) swabs and bronchoalveolar lavages (BAL).**

### **5.1 Objective**

The objective of this study was to compare the prevalence of *M. bovis* from NP and BAL sampling, using standard microbiological culturing techniques and a polymerase chain reaction assay (PCR), in healthy feedlot steers at 14 and 90 days on feed (DOF). This study is herein referred to as the **NP-BAL Study**. The *Mycoplasma bovis* isolates were also differentiated by AFLP and compared to isolates from two other studies (Pound-Maker and Necropsy).

### **5.2 Materials and Methods**

Three-hundred and ninety-two steer calves were purchased from local auction marts (n = 6) between October 18 and November 15, 2007. The animals weighed between 219 and 333 kg. All were routinely processed on entry to the feedlot, which included weighing and tagging each individual calf as well as administering a multivalent vaccine. Calves were then placed in 12 pens of 32-33 animals/pen. Three to five calves were randomly chosen from 8 pens and enrolled in the study.

The first nasopharyngeal swabs (NP) and bronchoalveolar lavages (BAL) were collected between 10 – 14 days post-arrival with sampling occurring on 3 separate days. Subsequently, the calves were resorted on December 7, 2007 and sampled again at 90 days on feed (DOF). The NP and BAL collection techniques have been previously described.

The NP swab was placed in Ames media for storage and the samples transferred from the U of S feedlot back to the veterinary college for processing. All NP and BAL samples were stored in a -82 °C freezer until culture; samples were stored no longer than one week prior to culture. A second set of NP and BAL samples were obtained at ~ 90 days on feed (DOF).

The culturing methods, PCR identification and strain differentiation techniques (AFLP) have all been previously described.

Prevalence was calculated by using the number of animals positive by PCR for *Mycoplasma bovis* divided by the total number of animals from which cultures were obtained, or 'total analysed'. Fisher's exact test was used to test for differences in the prevalence of *M. bovis* in the BAL and NP samples and across the 2 test periods. The Kappa statistic was used to analyze for the level of agreement between NP and BAL sampling techniques.

### 5.3 Results

Thirty-six calves were enrolled in the study. Culture results (Table 5.1) were available from all sampled animals; however, 3 animals were missed for BAL and NP and 4 were missed for BAL at the 90 DOF sampling. *Mycoplasma spp.* were more commonly recovered from the NP versus the BAL samples, which was true for both sampling periods. Appendix E contains two flow charts of the sampling procedures. Appendix F shows the individual animal culture results, NP and BAL samples, for days 14 and 90 DOF.

Table 5.1 Comparison of *Mycoplasma* culture results between NP swabbing and BAL at 14 and 90 DOF.

|                            | NP 14 DOF (%) | BAL 14 DOF (%) | NP 90 DOF (%) | BAL 90 DOF (%) |
|----------------------------|---------------|----------------|---------------|----------------|
| <b>Mycoplasma Positive</b> | 30 (83)       | 14 (39)        | 31 (94)       | 21 (72)        |
| <b>No Growth</b>           | 6 (17)        | 22 (61)        | 2 (6)         | 8 (28)         |
| <b>Total</b>               | 36            | 36             | 33            | 29             |

Table 5.2 summarizes the PCR results for all animals and provides the prevalence of *M. bovis*; PCR results were not available from all isolates.

Table 5.2 Comparison of PCR results between nasal swabbing and BAL of individual animals at 14 and 90 DOF

|                   | <b>M. bovis</b> | <b>Non-M. bovis species</b> | <b>No Growth</b> | <b>M. bovis Prevalence</b> |
|-------------------|-----------------|-----------------------------|------------------|----------------------------|
| <b>NP 14 DOF</b>  | 3               | 20                          | 6                | 10%                        |
| <b>BAL 14 DOF</b> | 1               | 10                          | 22               | 3%                         |
| <b>NP 90 DOF</b>  | 18              | 9                           | 2                | 62%                        |
| <b>BAL 90 DOF</b> | 7               | 14                          | 8                | 24%                        |

The prevalence of *M. bovis* increased 6 - 8 times over the 76 day study period. At 14 DOF the prevalence of *M. bovis* in the study group was 10% in the upper respiratory tract and 3% in the lungs. By 90 DOF, the prevalence increased to 62% in the upper respiratory tract and 24% in the lungs. The change in prevalence between 14 and 90 DOF for both NP ( $P < 0.01$ ) and BAL

( $P = 0.01$ ) samples were significantly different using McNemar's test. Kappa agreement between NP and BAL was not significant. There was poor agreement in the recovery of *M. bovis* from NP versus BAL at 14 DOF ( $K = 0.18$ ,  $P = 0.06$ ) and no agreement at 90 DOF ( $K = 0.08$ ,  $P = 0.3$ ).

#### 5.4 Conclusions

Samples were obtained from the deep within the nasal passages (nasopharyngeal swabs) and lung fields (bronchoalveolar lavages) of clinically normal calves at 14 days and 90 days after placement into a feedlot. The prevalence of *M. bovis* increased during the feeding period with regard to both the upper and lower respiratory tracts. Also, the prevalence of *M. bovis* was consistently higher in NP samples than BAL samples.

There was very poor agreement between the prevalence *Mycoplasma spp.* as determined by BAL and NP at either 14 or 90 DOF. In contrast, Allen *et al* (1991) found moderate agreement ( $K = 0.51$ ,  $P \leq 0.05$ ) between NP and BAL results; we may have gotten better agreement had we used a larger sample population. The lack of agreement may also indicate that *M. bovis* forms part of the normal nasal flora and does not necessarily mean that the bacteria will be found in the lung.

In conclusion, the prevalence of *M. bovis* increases significantly during the feeding period in both the upper and lower respiratory tracts. Unfortunately the prevalence of *M. bovis* in the lungs cannot be accurately predicted by the prevalence of *M. bovis* in the nasopharynx, and likely no conclusions of disease status may be made using NP samples alone.

The strain differentiation results are discussed in Chapter 8.

## **6. A survey of *Mycoplasma bovis* strains in western Canadian feedlot cattle.**

### **6.1 Objective**

The objectives of this study were the same as those in the original grant application, namely, to: 1) determine the prevalence of *Mycoplasma bovis* in fall-placed feedlot calves; 2) describe the ecology of mycoplasma within pens of feedlot calves; and 3) relate the genetic differences, species and strain variations, to pathogenicity in the field environment. This study is herein referred to as the **Pound-Maker study**.

### **6.2 Materials and methods**

Section 1 of this report discussed the amendments that were needed to the original Research Plan.

#### ***Fall of 2007***

On October 29, 2007, nasopharyngeal (NP) swabs were obtained from 325 cattle designated as Lot 142. The following day, October 31, an additional 325 calves were sampled, placed in a separate pen, and designated Lot 681. Over the next 3 months 22 calves from Lot 142 and 41 calves from Lot 681 were treated at least once for respiratory disease, at which time a second NP sample was taken by the 'treatment crew' and stored in a -20C freezer until it was picked-up by a WCVN clinician who returned the swab to the veterinary college for storage in a -82°C freezer. Pen level treatment rates of 6.7% (22/325) and 12.6% (41/325) were well below the historical treatment rates of 25%. Furthermore, only 5 animals in these 2 pens died; the expected number was 8 (5% of the "1<sup>st</sup> treatment pulls", which would be 5% of 163 (650 x 25%)).

#### ***Fall of 2008***

The decision was made to swab all the calves that were being housed at the U of S feedlot in the fall of 2008. These calves were split into two lots: Pound-Maker cattle and McKinnon cattle (Pound-Maker Feedyards has an agreement with the University wherein it will purchase calves and place them in the U of S feedlot for research purposes.) The McKinnon cattle were Dr. John McKinnon's and were involved in a feeding study along with the Pound-Maker cattle. It needs to be made clear that the 2007 calves were all sampled at Pound-Maker's feedlot, whereas

approximately half the 2008 calves were owned by Pound-Maker but were being fed at the U of S feedlot.

In total, 396 Poundmaker cattle were allocated to 12 pens, each having 33 head. There was another 288 McKinnon cattle that were allocated to 24 pens, each having 12 head/pen. A single NP sample was collected from all 684 head of cattle and then stored at in a -82°C freezer. All the cattle were sampled between November 11, 2008 and December 6, 2008. The calves were then monitored for disease and mortality. Unfortunately, only 11 animals (1.6%) were treated for respiratory disease.

Because of the very low number of animals that were treated, we elected to obtain a nasal swab on all the animals in March and May 2009; the second sample (May 200) was obtained for future studies.

### 6.3 Results

A NP swab was obtained from 650 calves in 2007 at time of entry into Pound-Maker's feedlot and from the 684 calves that entered the U of S feedlot in 2008. Of the 2007 calves, 56 were sampled at the time of treatment for respiratory disease. Thus, there were 56 paired samples: on-arrival swab and a treatment swab. In regards to the U of S cattle that were sampled in the fall of 2008, the incidence of disease was so low that these samples were not processed, hence this report is restricted to the cattle from the 2007 sampling at Pound-Maker.

The prevalence of *Mycoplasma* species cultured from nasal swabs taken from cattle that developed respiratory disease symptoms at Pound-Maker is reported in Table 6.1). *M. bovis* and *M. bovirhinis* were the primary species cultured. The number of animals positive for *M. bovis* was significantly higher at 1 week to 3 months than upon arrival. There was, however, no significant difference between the numbers of animals that had positive cultures for *M. bovirhinis* when sampled versus at time of treatment (Table 6.1). Four other species of *Mycoplasma* were also cultured, including, in order of prevalence: *M. ovipneumoniae*, *M. lagogenitalium*, *M. alkalescens*, and *M. cynos*. The number of animals in which multiple *Mycoplasma* spp. could be cultured increased from 7% at arrival to 21% upon treatment. There was also no correlation between the *M. bovis* status of the calves on arrival to later treatment for respiratory disease symptoms (Table 6.2).

A total of 138 isolates from 42 calves were differentiated by AFLP. These data have been summarized and reported in the final chapter of this report along with strain differentiation data from the NP-BAL and Necropsy studies.

Table 6.1 Number (%) of bacterial species cultured from nasal swab samples taken from feedlot steers on arrival at the feedlot and at treatment for respiratory disease 1 week to 3 months later.

| Sample Time  | Number Animals <sup>a</sup> | M. bovis | M. bovirhinis | Other Species | Multiple Species <sup>a</sup> | No Mycoplasma Growth |
|--------------|-----------------------------|----------|---------------|---------------|-------------------------------|----------------------|
| On arrival   | 56                          | 24 (41)  | 18 (32)       | 1 (2)         | 4 (7)                         | 17 (30)              |
| At treatment | 56                          | 38 (68)  | 11 (19)       | 14 (25)       | 12 (21)                       | 5 (9)                |

<sup>a</sup> Individual animals may be represented in more than one Mycoplasma group classification (column)

Table 6.2 Number (%) of NP samples either positive (+) or negative (-) for *Mycoplasma bovis* at the time of arrival into the feedlot and at the time of 1<sup>st</sup> treatment<sup>a</sup>.

|                                        | <i>M. bovis</i> positive<br>At 1 <sup>st</sup> treatment | <i>M. bovis</i> negative<br>At 1 <sup>st</sup> treatment |
|----------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| <i>M. bovis</i> positive<br>On-arrival | 17 (50%)                                                 | 4 (12%)                                                  |
| <i>M. bovis</i> negative<br>On-arrival | 10 (29%)                                                 | 3 (9%)                                                   |

<sup>a</sup>Insufficient data for either 'on-arrival' or 'at 1<sup>st</sup> treatment' groups for 22 animals therefore only 34 animals included in this table.

## 6.4 Conclusions

*Mycoplasma bovis* was isolated from ~40% of the calves' nasopharynx at the time of arrival into the feedlot, increasing to ~70% at the time of treatment. While other species of Mycoplasma were isolated, *M. bovis* was by far the most predominant isolate. This finding differed from the NP-BAL study where *Mycoplasma bovirhinis* was the most commonly isolated species, followed by *M. bovis*. And while the prevalence of *M. bovis* increased from the time of arrival to treatment ( $P = 0.008$ ), this was not true for *M. bovirhinis* ( $P = 0.11$ ), which was the second most common Mycoplasma isolate. Significantly, whether or not *M. bovis* could be isolated on-arrival was unrelated to whether it could or could not be isolated at the time of treatment ( $P = 0.55$ ). This is significant because it suggests that the presence or absence of *M. bovis* is not predictive of which calves will be treated for respiratory disease over the ensuing 3 months into the feeding period.



## **7. *Mycoplasma* spp. isolates from calves diagnosed at necropsy with chronic pneumonia and polyarthritis syndrome (CPPS).**

### **7.1 Objective**

To determine whether there were strain differences in the *Mycoplasma bovis* isolates obtained from infected lung tissue and septic joint(s) of calves that died of presumptive *M. bovis* infections. Secondly, to determine whether particular strains of *M. bovis* are more likely to be isolated across multiple feedyards. This study is herein referred to as the **Necropsy study**.

### **7.2 Materials and methods**

Approximately 10 veterinary clinics located in Saskatchewan and Alberta, known to have feedlot clients, were contacted in the summer of 2007 to see if they would participate in a 'necropsy' study. Specifically, veterinarians were asked to provide a lung sample and joint swab from feedlot calves that had lesions consistent with the chronic pneumonia and polyarthritis syndrome (CPPS). The goal was to obtain at least 40 samples from various feedlots and to use the AFLP technique for strain differentiation. To encourage participation, "necropsy kits" were sent-out to each clinic, which included swabs and containers for sample collection, data capture forms, and a video describing the case definition of CPPS and sampling technique. See Appendix G for a copy of the documentation that was sent to the veterinarians. Veterinarians were offered \$25 for every sample submitted.

### **7.3 Results**

Four veterinary practices participated in the necropsy study. Samples were collected between December 2007 and January 2009. As previously stated, the incidence of respiratory disease and CPPS was quite low in 2007 and this also appeared to be the case in 2008. However, we received 61 samples from 14 feedlots, 12 of which were located in Alberta. The JVD clinic was located in southern Alberta. Appendix H provides the individual animal culture results.

While veterinarians were asked to submit both lung tissue and a joint swab, this was not always done. In some cases only lung tissue was submitted and in 1 case only a joint swab was submitted. Table 7.1 shows the breakdown of submissions by sample type. Table 7.2 shows the samples that were positive for *Mycoplasma* species as well as the number of animals that were

positive for *M. bovis* by PCR. Because of logistical reasons not every *Mycoplasma spp.* isolate was subjected to the PCR test, rather only 29 of the *Mycoplasma spp.* isolates were typed, 25 of which were positive for *Mycoplasma bovis*, the other isolates were: *Paenibacillus chibensis*, and *M. alkalescens*.

Table 7.1 Summary of the necropsy submissions by veterinary clinic and feedlot.

| <b>Veterinary Clinic</b> | <b>Feedlot</b> | <b>Number of Animals</b> | <b>Number of lung samples</b> | <b>Number of joint swabs</b> |
|--------------------------|----------------|--------------------------|-------------------------------|------------------------------|
| JVD                      | T              | 10                       | 10                            | 9                            |
|                          | East           | 13                       | 13                            | 10                           |
|                          | West           | 10                       | 10                            | 10                           |
|                          | North          | 4                        | 4                             | 4                            |
|                          | RPL            | 2                        | 2                             | 1                            |
|                          | Home           | 1                        | 1                             | 0                            |
|                          | LL             | 4                        | 4                             | 3                            |
| VAHS                     | M              | 1                        | 1                             | 1                            |
|                          | N              | 4                        | 3                             | 2                            |
|                          | P              | 1                        | 1                             | 0                            |
| Kent                     | BWF            | 1                        | 1                             | 1                            |
|                          | Green          | 1                        | 1                             | 1                            |
| U of S                   | U of S         | 1                        | 1                             | 0                            |
|                          | L              | 8                        | 8                             | 5                            |
|                          | 14 feedlots    | Total = 62               | Total = 61                    | Total = 47                   |

Table 7.2 Summary of the culture results showing the number of lung tissue and joint swabs that were positive for *Mycoplasma spp.* and the number of animals in which either culture (lung or joint) was typed by PCR as a positive *M. bovis* isolate.

| Veterinary Clinic | Feedlot     | Positive Lung <i>Mycoplasma spp.</i> | Positive Joint <i>Mycoplasma spp.</i> | Number typed to <i>M. bovis</i>          |
|-------------------|-------------|--------------------------------------|---------------------------------------|------------------------------------------|
| JVD               | T           | 10/10                                | 9/9                                   | 2/2                                      |
|                   | East        | 12/13                                | 9/10                                  | 3 /4                                     |
|                   | West        | 9/10                                 | 6/10                                  | 8/8                                      |
|                   | North       | 4/4                                  | 4/4                                   | 3 /4                                     |
|                   | RPL         | 2/2                                  | 1/1                                   | 2/2                                      |
|                   | Home        | 1/1                                  | 0/0                                   | 1/1                                      |
|                   | LL          | 4/4                                  | 1/3                                   | 0/0                                      |
| VAHS              | M           | 1/1                                  | 0/1                                   | 1/1                                      |
|                   | N           | 3/4                                  | 1/2                                   | 2/4                                      |
|                   | P           | 1/1                                  | 0/0                                   | 1/1                                      |
| Kent              | BWF         | 1/1                                  | 1/1                                   | 1/1                                      |
|                   | Green       | 1/1                                  | 0/1                                   | 0/0                                      |
| U of S            | U of S      | 1/1                                  | 0/0                                   | 1/1                                      |
|                   | L           | 7/8                                  | 4/5                                   | 0/0                                      |
|                   | 14 feedlots | Total positive<br>= 57/61 (93.4%)    | Total positive<br>= 36/47 (76.6%)     | Total <i>M. bovis</i><br>= 29/29 (86.2%) |

## 7.4 Conclusions

*Mycoplasma bovis* was routinely cultured from the lungs, and in many cases the joints, of animals diagnosed with CPPS. Isolates that were positively identified by PCR as *Mycoplasma bovis* were subsequently analyzed by AFLP for strain differentiation. These results will be discussed in the next section.

## **8. Summary of strain differentiation (AFLP) analysis of samples from the NP-BAL study, Pound-Maker feedlot study, and the Necropsy study.**

### **8.1 Objective**

The objective of this study was compare, using the AFLP typing technique, isolates of *M. bovis* obtained from 3 different populations of cattle: 1) isolates from the previously discussed NP-BAL study; 2) isolates derived from nasopharyngeal swabs taken from cattle upon arrival at Pound-Maker and again when the cattle were treated for respiratory disease; and 3) isolates obtained from lung tissue samples and swabs of joints obtained from cattle that died or were euthanized because of chronic pneumonia and polyarthritis syndrome (CPPS).

### **8.2 Materials and Methods**

The sampling of the 3 sets of cattle has been previously described.

### **8.3 Results**

#### **Culture results**

##### ***NP-BAL (U of S Healthy cattle) Study***

Multiple *Mycoplasma* species were cultured from between 6-11% of healthy University of Saskatchewan tested and there was no significant difference between the number of animals in which multiple species could be cultured at 14 vs 90 DOF in the either the NP or BAL samples. *Mycoplasma bovis* was the second most commonly cultured *Mycoplasma* species after *M. bovirhinis* (Table 8.1). A significant increase in *M. bovis* positive animals was seen at 90 DOF compared to 14 DOF NP ( $P = 0.002$ ) and BAL ( $P = 0.01$ ). Only two *Mycoplasmas* species other than *Mycoplasma bovis* and *Mycoplasma bovirhinis* were cultured from University of Saskatchewan feedlot cattle, namely *Mycoplasma alkalescens* and *Mycoplasma bovocoli*. There was no correlation between the calves' *M. bovis* status at 14 and 90 DOF (Fisher's Exact test, two-tailed:  $P = 1.0$ ). There was also a very weak association between the NP and BAL results (Fisher's Exact test, two-tailed:  $P = 0.09$ ).

Table 8.1. Breakdown, number and percent (%), of the NP-BAL study calves by *Mycoplasma* isolate, day of sampling (14 and 90 DOF), and type of sample (NP and BAL).

| Sample Type          | Days on Feed | Number of Animals <sup>b</sup> | M. bovis <sup>c</sup> | M. bovirhinis | Other Species | Multiple Species | No <i>Mycoplasma</i> Growth |
|----------------------|--------------|--------------------------------|-----------------------|---------------|---------------|------------------|-----------------------------|
| NS                   | 14           | 36                             | 3 (8)                 | 19 (53)       | 4 (11)        | 3 (8)            | 13 (36)                     |
| NS                   | 90           | 36                             | 14 (39)               | 15 (42)       | 1 (3)         | 4 (11)           | 10 (28)                     |
| P value <sup>d</sup> |              |                                | 0.002                 | 0.35          | 0.17          | 0.69             | 0.45                        |
|                      |              |                                |                       |               |               |                  |                             |
| BAL                  | 14           | 36                             | 1 (3)                 | 11 (31)       | 1 (3)         | 2 (6)            | 25 (69)                     |
| BAL                  | 90           | 36                             | 7 (19)                | 17 (47)       | 0 (0)         | 2 (6)            | 14 (39)                     |

<sup>b</sup> Individual animals may be represented in more than one *Mycoplasma* group classification (column).

<sup>c</sup> Bacterial species assignment based on partial 16S rRNA gene sequence analysis.

#### ***Pound-Maker study***

Table 8.2 shows the prevalence of *Mycoplasma* species cultured from NP swabs taken from Pound-Maker cattle that were being treated at the time of sampling for respiratory disease. While *M. bovis* and *M. bovirhinis* were again the primary species cultured, in this scenario *M. bovis* was the most common isolate. The number of animals positive for *M. bovis* upon was significantly higher at treatment than at arrival. The number of animals in which multiple *Mycoplasma* species could be cultured also significantly increased from 7% at the time of arrival to 21% upon treatment 1 week to 3 months later. There was no correlation between the *M. bovis* status of the cattle on arrival at the feedlot and 1 week to 3 months later upon treatment for respiratory disease symptoms (Fisher's Exact test, two-tailed:  $P = 1.0$ ).

Table 8.2 Breakdown by number (%) Pound-Maker calves by *Mycoplasma* isolate and by day of entry versus at time of first treatment.

| Sample Time  | Number of Animals <sup>a</sup> | M. bovis | M. bovirhinis | Other Species | Multiple Species <sup>a</sup> | No <i>Mycoplasma</i> Growth |
|--------------|--------------------------------|----------|---------------|---------------|-------------------------------|-----------------------------|
| On arrival   | 56                             | 24 (41)  | 18 (32)       | 1 (2)         | 4 (7)                         | 17 (30)                     |
| At treatment | 56                             | 38 (68)  | 11 (19)       | 14 (25)       | 12 (21)                       | 5 (9)                       |

<sup>a</sup> Individual animals may be represented in more than one *Mycoplasma* group classification (column).

### Strain differentiation

Only animals positive for *M. bovis* were used for strain differentiation and some of these were excluded due to either lack of culture material, insufficient DNA or failure of AFLP to produce a fingerprint for one of the sampling periods. Strain differentiation was done on 63 isolates from 20 NP-BAL study calves, 138 isolates from 42 Pound-Maker calves, and on *M. bovis* isolates from 19 carcasses.

AFLP strain differentiation and cluster analysis performed on *M. bovis* strains isolated from the 19 necropsied carcasses revealed 3 main clusters, Groups or clusters A, B and C (Figure 8.1). Isolates within each cluster show a similarity score of greater than 75% based on the dice coefficient. Strain A was isolated from both the lung and joint samples of the majority of animals (Table 8.3); however, isolation of multiple strains was not uncommon in both the lung and joint samples. Both joint and lung samples were available from only 7 animals and therefore this group is too small to be statistically significant. However 5 of these animals were colonized by multiple strains and 3 of these animals showed disagreement in strains isolated from the lung and those isolated from the joint.

Table 8.3 Number (percent) of cattle in the Necropsy study with *Mycoplasma bovis* isolates from lung and joint that cluster in groups A, B, and C, and thee number with multiple strains (clusters) within the same animal.

| Sample Type | Number of Animals <sup>a</sup> | Group A | Group B | Group C | Multiple Clusters |
|-------------|--------------------------------|---------|---------|---------|-------------------|
| Lung        | 17                             | 12 (71) | 5 (29)  | 5 (29)  | 5 (29)            |
| Joint       | 8                              | 8 (100) | 1(13)   | 2 (25)  | 3 (38)            |

<sup>a</sup> Individual animals may be represented in more than one cluster classification (column).

Cattle included in 'Multiple Strains' column not also included in 'Strain A', 'Strain B' and 'Strain C' columns.

AFLP strain differentiation was completed for 62 isolates representing 20 cattle from the NP-BAL study and 138 isolates from 42 Pound-Maker cattle. The AFLP fingerprints from the Pound-Maker study clustered almost entirely with Group A (Figure 8.2), with the exception of 1 isolate that clustered in Group B. In contrast, most of the NP and BAL isolates (all healthy cattle) clustered with Group B (Figure 8.3), with some clustering within Group A. None of the isolates from either the NP-BAL or Pound-Maker study clustered with Group C.

There was no difference in the clustering pattern (Group A, B, or C) of the *M. bovis* isolates derived from the lungs versus the joints of the 19 necropsied cattle ( $P = 0.54$ ), see Table 8.3. Similarly, there was difference in clustering of *M. bovis* isolates obtained from the Pound-Maker cattle at the time of entry to the feedlot and treatment ( $P = 0.42$ ), see Table 8.4. With respect to the NP-BAL cattle, there was no difference in the clustering of the 14 and 90 DOF isolates ( $P = 0.85$ ; Table 8.5), or in the clustering of the NP versus BAL isolates ( $P = 0.91$ ; Table 8.6). What these data suggest is that isolates on entry into the feedlot are similar to those at treatment; isolates obtained at 14 DOF are similar to those obtained at 90 DOF; and the NP and BAL isolates are similar.

Table 8.4. Number (and percent) of *Mycoplasma bovis* isolates obtained from the Pound-Maker cattle that clustered into Groups A, B or C.

| Sample Time  | Number of Animals <sup>a</sup> | Cluster A | Cluster B | Cluster C | Multiple Clusters |
|--------------|--------------------------------|-----------|-----------|-----------|-------------------|
| On Arrival   | 25                             | 25 (100)  | 0 (0)     | 0 (0)     | 0 (0)             |
| At Treatment | 38                             | 38 (100)  | 1 (3)     | 0 (0)     | 1 (3)             |

<sup>a</sup> Individual animals may be represented in more than one cluster classification (column).

Table. 8.5 Number (and percent) of *Mycoplasma bovis* isolates that were obtained at 14 and 90 DOF in the NP-BAL study that clustered into Groups A, B or C.

| Sample Time | Number of Animals <sup>a</sup> | Cluster A | Cluster B | Cluster C | Multiple Clusters |
|-------------|--------------------------------|-----------|-----------|-----------|-------------------|
| 14-DOF      | 5                              | 2 (40)    | 3 (60)    | 0 (0)     | 0 (0)             |
| 90-DOF      | 12                             | 6 (50)    | 11 (92)   | 0 (0)     | 5 (42)            |

<sup>a</sup> Individual animals may be represented in more than one cluster classification (column).

Table. 8.6 Number (and percent) of *Mycoplasma bovis* isolates obtained from the NP and BAL samples that clustered into Groups A, B or C.

| Type of Sample | Number of Animals <sup>a</sup> | Cluster A | Cluster B | Cluster C | Multiple Clusters |
|----------------|--------------------------------|-----------|-----------|-----------|-------------------|
| NP             | 13                             | 5 (38)    | 11 (85)   | 0 (0)     | 3 (23)            |
| BAL            | 7                              | 3 (43)    | 6 (86)    | 0 (0)     | 2 (29)            |

<sup>a</sup> Individual animals may be represented in more than one cluster classification (column).



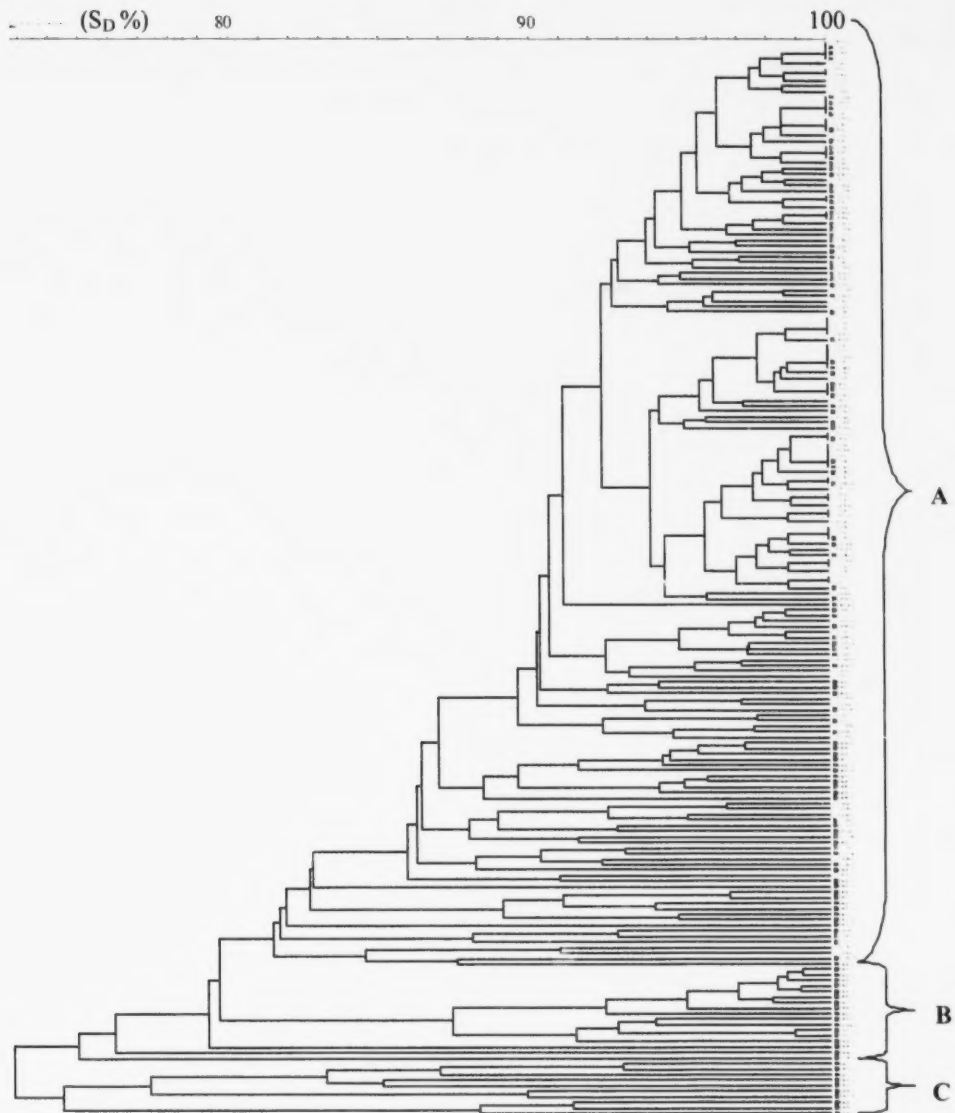


Figure 8.2 Dendrogram based on AFLP banding patterns for *Mycoplasma bovis* isolates cultured from nasal swabs (NP) collected at Pound-Maker feedyards from the lung and joint isolates obtained from the Necropsy study. Three clusters are labelled A, B and C based on the clustering of post-mortem samples shown in Figure 3.2. Green represents necropsy strains, yellow represents isolates from asymptomatic calves on arrival at feedlot and blue represents isolates taken from these cattle 1 week to 3 months later upon treatment for symptoms of respiratory disease.

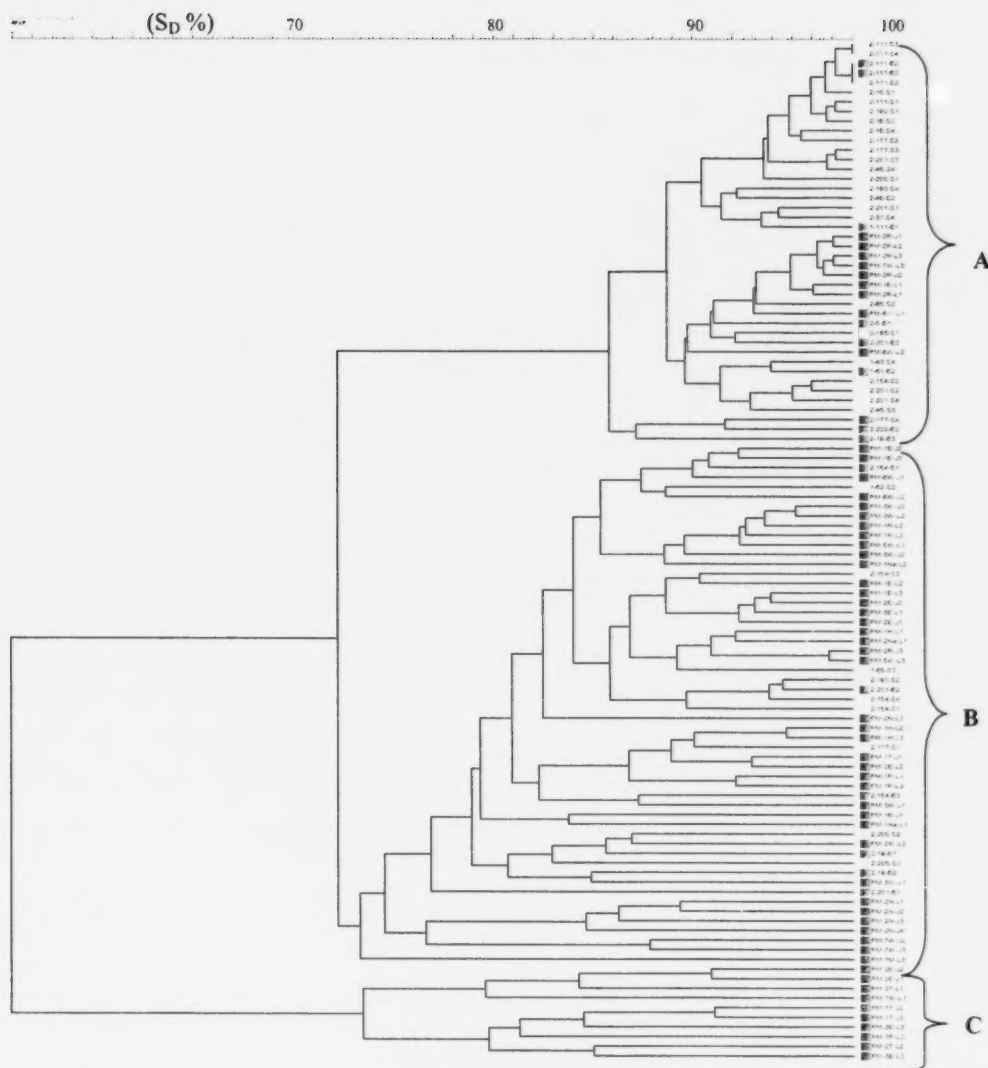


Figure 8.3. Dendrogram of *Mycoplasma bovis* strains isolated from nasopharyngeal swabs (NP) and bronchioalveolar lavages (BAL) of healthy feedlot cattle at the University of Saskatchewan feedlot. Three clusters are labelled A, B and C based on the clustering of the necropsied cattle. Green represents necropsy-derived strains, blue represents BAL isolates, and yellow represents NP isolates.

## 8.4 Discussion

Increased colonization of the nasopharynx by *Mycoplasma* spp., particularly *M. bovis*, increased with the number of days on feed (DOF); however, there was no correlation between an individual animal's *M. bovis* status early in the feedlot and at later time points. Several published studies support this positive correlation between *Mycoplasma* colonization and the number of DOF (Allen *et al*, 1992; Rosendal and Martin *et al*, 1986). Perhaps the increased colonization is related to the length of time calves are exposed to shedders of *Mycoplasma* (Alexander *et al*, 1989; Ribble *et al*, 1995).

*Mycoplasma bovis* is highly correlated with chronic pneumonia and polyarthritis syndrome in feedlot scenarios, but can also be isolated from the respiratory passages of healthy animals (Allen *et al*, 1991; 1992; Booker *et al*, 2008; Gagea *et al*, 2006; Haines *et al*, 2001; Hirose *et al*, 2003; Maeda *et al*, 2003; Radaelli *et al*, 2008; Rodríguez *et al*, 1996; Rosendal and Martin, 1986; Sachse *et al*, 2009; ter Laak *et al*, 1992b; Thomas *et al*, 2002; Wiggins *et al*, 2007).

It was not usual to culture multiple *Mycoplasma* spp. isolates from the same animal, and this did not appear to vary with the number of DOF. Thomas *et al* (2002) also observed that while one or two *Mycoplasma* spp. were generally predominant in the feedlot, it was not uncommon for a single animal to harbour several species. This observation suggests that while there may be some competition between closely related *Mycoplasma* species it is possible for them to coexist in the same animal. While only 7 animals had data for both lung and joint samples, multiple strains could be identified in a single tissue for 2 of these animals and different strains were isolated in the lung versus the joint in 3 animals. This is consistent with an earlier study in which genetically distinct *M. bovis* strains were isolated from different body sites within a single animal (Biddle *et al*, 2005). Further research with strain differentiation in multiple body sites from larger groups of animals may provide a better understanding of this strain discrepancy within and between sampling locations

One of the objectives of the current study was to attempt to distinguish between virulent and avirulent strains of *M. bovis* by employing molecular strain differentiation to compare populations in CPPS afflicted and healthy cattle. Several molecular methods for *M. bovis* strain differentiation have been reported, each with varying degrees of specificity, sensitivity, reproducibility, speed, technical and labour requirements (Beier *et al*, 1998; Biddle *et al*, 2005; Butler *et al*, 2001; Fox, 2008; Kusiluka *et al*, 2000; McAuliffe *et al*, 2004; 2008; Miles *et al*, 2005). RAPD was initially chosen as the method of strain differentiation for this study as previous literature had found RAPD to be as sensitive, and specific as other methods, but with fewer technical and time requirements (McAuliffe *et al*, 2004, Williams *et al*, 1990). However, in

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the current study, RAPD methodology showed extremely poor reproducibility. Previous research did cite some reproducibility problems, but it appeared even with modifications to the original protocols the reproducibility was much lower than anticipated from this earlier research (Cherry *et al*, 2006; Cousin-Allery *et al*, 2000; Feberwee *et al*, 2005; Mettifogo *et al*, 2006; McAuliffe *et al*, 2004; Stakenborg *et al*, 2006). The low reproducibility made RAPD an unreliable method for strain differentiation in this study. AFLP methodology employed here was based on Kokotovic *et al* (1999) and produced reproducible banding patterns with greater than 92% similarity score based on dice coefficient for duplicate determinations. While this reproducibility is somewhat less than the identical densitometric curves reported by Kokotovic *et al* (1999) when duplicate samples were analyzed, the result is not surprising given the relatively complex multi-step AFLP protocol. Furthermore, this level of similarity for duplicate samples provided confidence in identifying genetically unique clusters or strains of *M. bovis* based on similarity scores that were less than 75%.

The necropsy animals came from 10 different feedlots in Alberta and Saskatchewan and therefore represent a diverse group. When strains were differentiated in CPPS clinical pathology necropsy lung and joint samples, three main clusters (A, B and C) were identified. Comparable studies found similar results in that relatively few distinct clusters were identified in cattle from a diverse geographical sampling (Kusiluka *et al*, 2002; McAuliffe *et al*, 2004). Several studies have demonstrated that much of the genetic variation between *M. bovis* isolates is in repetitive non-coding regions of the genome (McAuliffe *et al*, 2008; Miles *et al*, 2005). Therefore the differentiations made by molecular strain typing techniques may not cluster the isolates by expressed genes, but by these non-coding regions, and therefore may not be sensitive enough to differentiate strains based on virulence.

When the strains from cattle in the NP-BAL and commercial feedlot studies were compared to those from the CPPS necropsied animals, no new strains clusters were identified. The strains identified in the Pound-Maker feedlot from cattle treated for respiratory disease clustered mainly with Group A, while the strains identified in the NP-BAL study involving seemingly healthy cattle clustered mainly with Group B, with some isolates clustering with strain Group A. None of the NP-BAL or Necropsy study isolates clustered within Group C.

There were differences in the strains that colonized the nasopharynx versus the lower lung field. Several other studies have observed more *M. bovis* colonization in the nasal passage of healthy animals than the lower respiratory tract, indicating that variable virulence between strains may be responsible for the ability to colonize the lower respiratory tract (Allen *et al*, 1992; Catry *et al*, 2008). While the difference in *M. bovis* colonization between the upper and lower

respiratory tract could be a host dependent outcome, it may be further suggest that AFLP is not sensitive enough to differentiate strains based on variability between functional genes responsible for pathogenicity.

Two of the three strain clusters identified with AFLP in the necropsy group could also be isolated from healthy animals and those with undifferentiated respiratory disease. It is unknown whether the current molecular strain differentiation methodologies are not sensitive enough to distinguish these strains based on virulence genes or whether the ability of *M. bovis* to cause disease in the field is dependent on the host immune response or presence of other pathogens. Further research to identify potential pathogen associated genes so that differentiations between strains can be made based on differences in these specific areas of the genome.

## Appendices

## **Appendix A: Proposed Research Plan as provided in the grant application.**

### **Year 1 (Fall of 2007)**

Approximately 4 pens (1200 calves) of fall-placed calves, averaging 400-650 pounds, will be enrolled in the study. Calves will be sourced from auctions and directly from farms, with breeds being representative of the calves on feed in western Canada. All calves on entry will be identified by a unique tag (feedlot tag). Within 5 days of entry, a shielded colo-rectal swab designed for use in humans will be used to obtain a deep bronchial swab. Research has shown that the bacterial flora resident in the nasal cavities are poorly correlated to what is growing in the bronchi of the lungs, hence the need for the colo-rectal swabs. Swabs will be individually identified and samples will be placed in a refrigerator until they can be transported back to the WCVU, where they will be stored in liquid nitrogen (-86C). Previous research has shown that mycoplasma is extremely hardy and can be maintained for many months in liquid nitrogen.

Based on historical data, approximately 25% (300) of all feedlot calves are treated for undifferentiated bovine respiratory disease within the first few weeks after arrival ("first pull" calves). A 2nd transbronchial swab will be taken at the time of first treatment and a 3<sup>rd</sup> if the calf relapses again and enters the "chronic" pen. Previous ADF-funded research shows that approximately 5% (15 animals) of the first pull calves will either die or be euthanized on account of CPPS. Postmortem examinations will be conducted on all cattle to confirm the diagnosis and whenever possible a swab (or lung tissue) will be collected.

Upon completion of the study, the stored samples from all calves that were positively diagnosed as having CPPS (cases) will be subjected to a PCR technique known as T-RFLP. This is a variation on the previously published techniques but one that we believe will allow us to both type the mycoplasma by species (*bovis*, *dispar*, etc.) and to detect slight genetic differences that allow us to discern strain differences within the *Mycoplasma bovis* isolates. We expect this technique to be much more rapid and economical than current methods for culturing and typing. However, swabs that test positive on T-RFLP will be sent for culture so that additional genetic material is obtained for additional testing; an aliquot of the sample will also be archived for potential future testing.

Calves that are diagnosed at necropsy as having *Mycoplasma bovis* (CPPS) will represent the "cases". Two additional sets of "controls" will be matched 2:1 with the cases. Swabs from the first set of controls will come from those calves who were swabbed on entry but never went on to develop disease. Thus, we are comparing calves that were swabbed on entry and went on to die of mycoplasma (cases), to calves that had mycoplasma on entry into the feedlot but were never

treated. Because all the testing is done retrospectively, we can identify these calves at the end of the study using Pound-Makers computerized health records. The calf ID tag will then be matched to the swab, which has been stored in liquid nitrogen. Samples from the second set of controls will be obtained once the chronic pen is full. Typically, this pen will accumulate calves over time, we will keep track of the number of calves in the pen and then pull a equal number of healthy calves from the same pen that each calf came from. These healthy calves will be swabbed and the sample maintained in liquid nitrogen.

While over 1500 swabs will be collected, only about 150 swabs will be processed. The reason for having to obtain so many samples is because we cannot predict on entry which calves will get sick, which will be treated, and which will die. Sampling at each treatment will provide the basis ecological data. That is, we will know the prevalence of the various *Mycoplasma* spp., as well as the strains of *Mycoplasma bovis* at entry. Repeat sampling at 1<sup>st</sup> and 2<sup>nd</sup> pulls will allow us to see if the certain strains are more competitive than others. Some people have suggested that CPPS is an iatrogenic or "man-made" disease and that its increase in prevalence is a result of increased use in antimicrobials that destroy the nature flora of the upper respiratory system. Sampling before and after treatment may provide some insight into this theory. Finally, sampling the calves in the chronic pen should show unequivocally whether a certain strain(s) is associated with a higher incidence of CPPS. If so, then we can tentatively conclude that some strains may be more pathogenic than others.

All control samples will be processed at the same time as the case samples. The microbiologist and personnel involved in the genotyping will be blinded as to the treatment and control groups. Once all the mycoplasma cultures have been typed, they will be entered into a software program such as BioNumerics (Applied Maths), which will calculate the similarity between strains and provide a dendogram, or graphical output of all the strains. This analysis will allow us to determine how many clusters or lines of mycoplasma were resident in the calf population. Further statistical analyses will be done to describe the epidemiology of the strains. The origins of the calves (strains) will also be plotted using geographical software. This will provide a visual summary as to whether strains cluster in geographical areas of the province, and if so, then future epidemiological studies may be required to determine what risk factors are associated with the disease. Lastly, a statistical analyses will be done to determine if a certain strain, or strains, or organism were more closely correlated with morbidity and mortality. If so, then this would suggest that some strains may be more pathogenic than others.

Flow Chart for sampling.

4 pens (300/pen) of at risk calves = 1200 samples  
25% Pulled for 1st Treatment = 300 samples  
20% of 1st Pulls retreated (2<sup>nd</sup> pulls) = 60 samples  
3<sup>rd</sup> pull (Enter chronic pen)  
5% of 1st Pulls die = 15 samples

Number of samples processed from cases =  $15 \times 4 = 60$

Number of samples processed from two control groups = ~140

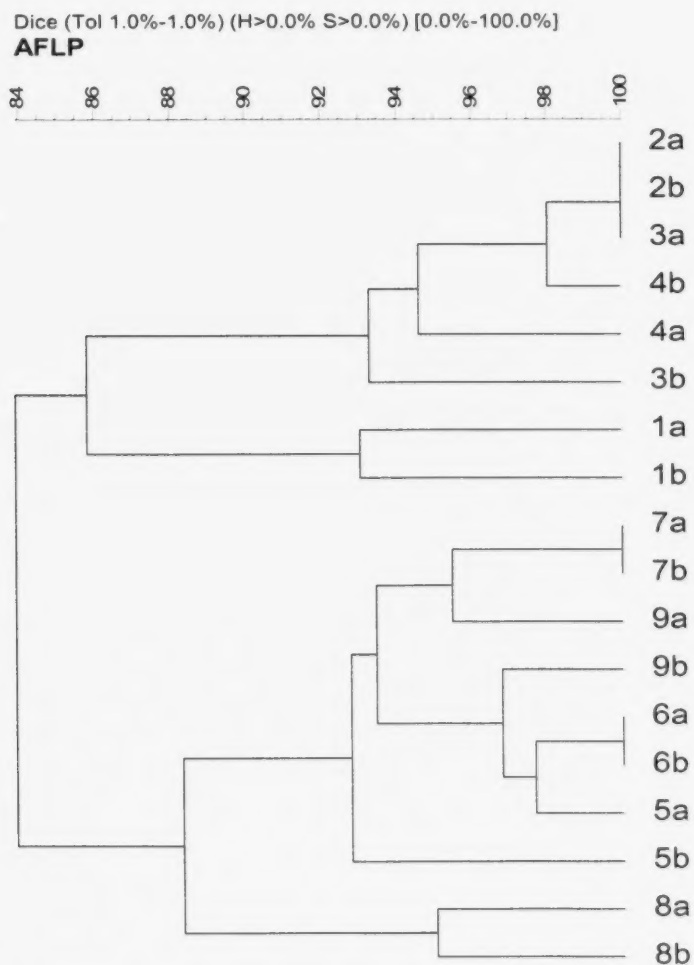
Total number of samples processed = 200

#### Year 2

The same experiment will be repeated in Year 2 (Fall of 2008). Again 4 pens of calves will be enrolled in the study. Slight modifications to the protocol may be made, if they are justified by the results from Year 1. However, one of the reasons for splitting the study is to examine year to year differences in prevalence and strain pathogenicity.

## Appendix B: Validation of the repeatability of the AFLP assay.

The following dendrogram represents the genetic relationship between **duplicate** *Mycoplasma bovis* strains isolated from feedlot cattle. It was produced by comparing banding patterns in the Bionumerics v.5.1 software with the Dice similarity coefficient  $S_D$  (%) using the unweighted pair group method. Nine samples were tested (1 – 9) and the duplicates labeled “a” and “b”.



## **Appendix C: SOPs for collecting nasopharyngeal (NP) samples and trans-tracheal washes (TTW).**

### **Procedure of nasopharyngeal swabs:**

Deep nasal swabs will be obtained using a 22" guarded cotton-tipped swab. The animal will be restrained in a head gate and the swab introduced through the nostril and advanced to the back of nasal cavity. At this point the guard on the swab will be withdrawn to expose the swab. A gentle twisting motion is sufficient to swab the nasal cavity. The guard will then be advanced to cover the tip and the entire unit removed.

### **SOP for Performing Trans-tracheal Wash (TTW) on Feedlot Steers:**

**Purpose:** To provide instructions for performing trans-tracheal wash as an ante-mortem diagnostic tool in feedlot steers.

**Materials:**

- a. Restraint - rope halter, chute, sedation (xylazine 0.1mg/kg IM or IV)
- b. Aseptic preparation of site – clippers, chlorhexidine scrub, alcohol, 4x4 gauze
- c. Local anesthesia – lidocaine, 6cc syringe, 20 gauge 1.5" needle
- d. Procedure – sterile gloves, #15 blade, 8 gauge needle, 5 french urinary catheter, 20 cc syringe, sterile physiologic saline
- e. Sample collection – red top blood tube

**Procedure:**

1. Restrain animal in a chute with a rope halter securely tying the head straight forward with the neck in slight extension.
2. Sedate the animal with xylazine (0.1mg/kg IV or IM)
3. Clip a an area of the midline of the neck, 6 inches above the thoracic inlet. Ensure that your site selection enables the person performing the procedure to be in a safe location with their

hands free from the head gate and their head away from the animals head.

4. Aseptically prepare the site.

5. Locally block the site using lidocaine.

6. Make a stab incision through the skin using a scalpel.

7. Insert the 8 gauge 1.5" needle through the stab incision and push it through the tracheal wall between tracheal rings. *Careful: Be sure to angle the needle down the trachea and be very careful not to penetrate the opposing side of the trachea.*

8. Insert a urinary catheter through the 8 gauge needle.

9. *Careful:* Remove the 8 gauge needle to ensure that the catheter is not cut off in the trachea.

10. Instill 20cc sterile physiologic saline and immediately withdraw a sample, moving the catheter slowly up and down the trachea. Another 20cc of sterile physiologic saline may be instilled if an adequate sample was not obtained. *Note: Do not exceed 1ml/kg body weight. Discontinue if animal becomes stressed.*

11. Place TTW sample into a red top blood tube. *Note: An adequate sample is equivalent to 2 mls of TTW fluid.*

12. Remove the 5 French catheter from the neck.

13. Close stab incision with 1-2 tissue staples.

14. Monitor animals post procedure for any signs of distress and local infection or cellulitis at the TTW site.

## Appendix D: Inventory of Preliminary Study samples

| U of S (Pound-Maker calves) |           |       |     |               |              |
|-----------------------------|-----------|-------|-----|---------------|--------------|
| Sample No                   | Animal ID | Nasal | TTW | Cultured - NS | Cultured-TTW |
| 1                           | 136-156   | 3x    |     | M. spp        |              |
| 2                           | -166      | 3x    |     | M. spp        |              |
| 3                           | 161       | 3x    | y   | M. spp        |              |
| 4                           | 179       | 3x    | y   | M. spp        |              |
| 5                           | 182       | 3x    | y   | Neg           |              |
| 6                           | 175       | 3x    | y   | Neg           |              |
| 7                           | 184       | 3x    | y   | Neg           |              |
| 8                           | 151       | 3x    | y   | M. spp        |              |
| 9                           | 160       | 3x    | y   | M. spp        |              |
| 10                          | 176       | 3x    | y   | M. spp        |              |
| 11                          | 155       | 3x    | y   | M. spp        |              |
| 3-TTW                       |           |       |     |               | M. spp       |
| 4-TTW                       |           |       |     |               | M. spp       |
| 5-TTW                       |           |       |     |               | No           |
| 6-TTW                       |           |       |     |               | M. spp       |
| 7-TTW                       |           |       |     |               | M. spp       |
| 8-TTW                       |           |       |     |               | M. spp       |
| 9-TTW                       |           |       |     |               | M. spp       |
| 10-TTW                      |           |       |     |               | No           |
| 11-TTW                      |           |       |     |               | No           |
| "W" Feedlot                 |           |       |     |               |              |
| Sample No                   | Animal ID | Nasal | TTW | Cultured - NS | Cultured-TTW |
| W001                        | D293      | 3x    |     | Neg           |              |
| W002                        | 39        | 2x    |     | Neg           |              |
| W003                        | 168       | 3x    |     | Neg           |              |
| W004                        | 168       | 3x    |     | M. spp        |              |
| W005                        | no tag    | 2x    |     | Neg           |              |
| W006                        | D54       | 2x    |     | Neg           |              |
| W007                        | Heifer    | 2x    |     | M. spp        |              |

|      |              |    |  |        |  |
|------|--------------|----|--|--------|--|
| W008 | I362         | 2x |  | Neg    |  |
| W009 | N149         | 2x |  | Neg    |  |
| W010 | W307         | 2x |  | M. spp |  |
| W011 | D129         | 2x |  | Neg    |  |
| W012 | Pink 127     | 2x |  | Neg    |  |
| W013 | I285         | 2x |  | Neg    |  |
| W014 | F282         | 2x |  | M. spp |  |
| W015 | W156         | 3x |  | Neg    |  |
| W016 |              | 3x |  | M. spp |  |
| W017 |              | 3x |  | Neg    |  |
| W018 |              | 2x |  | Neg    |  |
| W019 |              | 2x |  | Neg    |  |
| W020 |              | 2x |  | Neg    |  |
| W021 | (postmortem) | 3x |  | Neg    |  |

“H” Feedlot

| Sample No | Animal ID | Nasal | TTW | Cultured - NS | Cultured-TTW |
|-----------|-----------|-------|-----|---------------|--------------|
| H001      |           | 2x    |     | Neg           |              |
| H002      |           | 2x    |     | M. spp        |              |
| H003      |           | 2x    |     | M. spp        |              |
| H004      |           | 2x    |     | Neg           |              |
| H005      |           | 2x    |     | Neg           |              |
| H006      |           | 2x    |     | M. spp        |              |
| H007      |           | 2x    |     | M. spp        |              |
| H008      |           | 2x    |     | M. spp        |              |
| H009      |           | 2x    |     | Neg           |              |
| H010      |           | 2x    |     | Neg           |              |

“P”

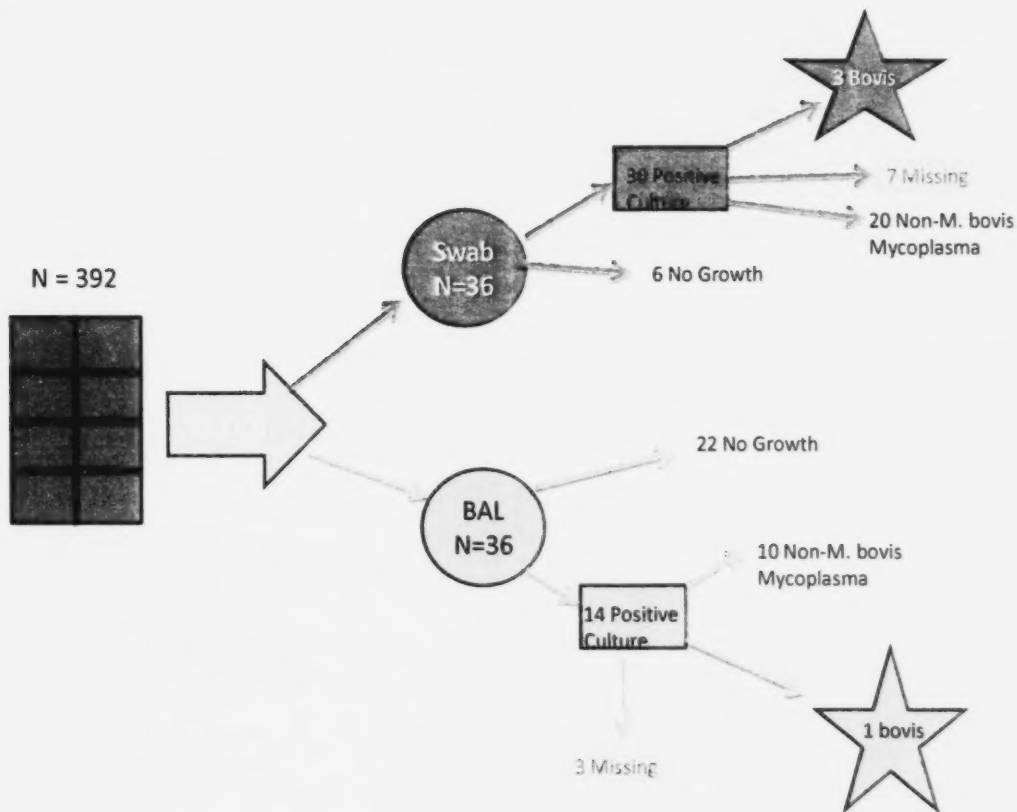
Feedlot

| Sample No | Animal ID | Nasal | TTW | Cultured - NS | Cultured-TTW |
|-----------|-----------|-------|-----|---------------|--------------|
| P001      |           | 2x    |     | Neg           |              |
| P002      |           | 2x    |     | M. spp        |              |

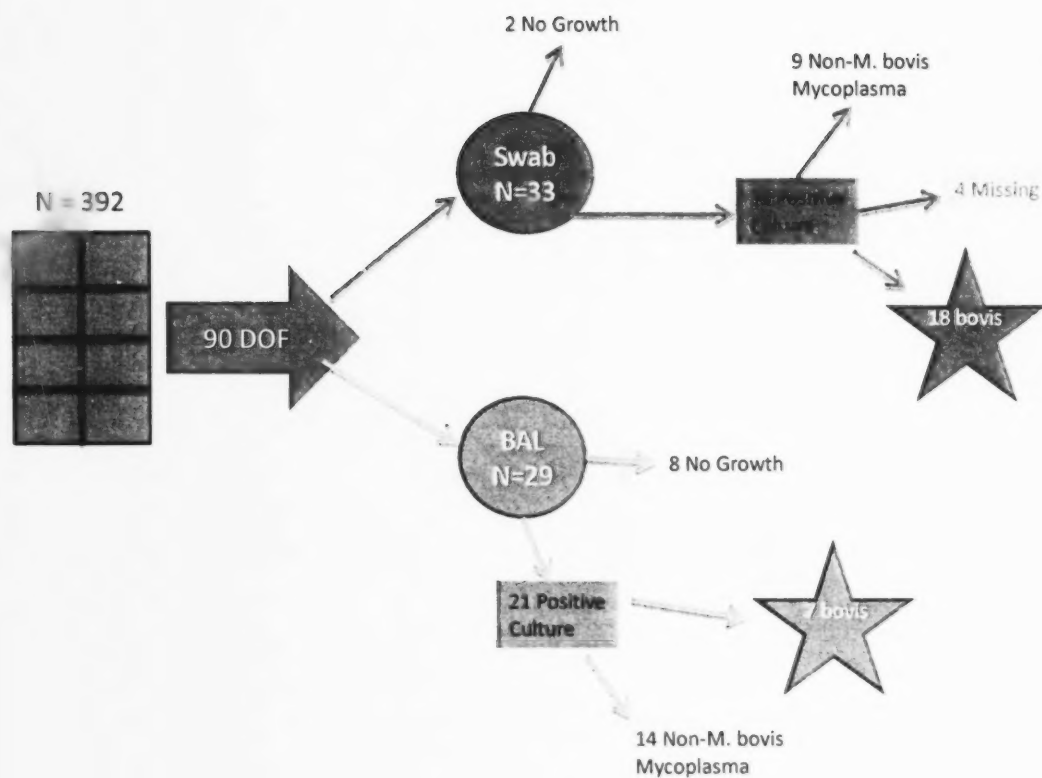
| P003         |             | 2x    |     | Neg           |              |
|--------------|-------------|-------|-----|---------------|--------------|
| P004         |             | 2x    |     | Neg           |              |
| P005         |             | 2x    |     | Neg           |              |
| P006         |             | 2x    |     | Neg           |              |
| P007         |             | 2x    |     | M. spp        |              |
| P008         |             | 2x    |     | Neg           |              |
| P009         |             | 2x    |     | M. spp        |              |
| P010         |             | 2x    |     | M. spp        |              |
| P011         |             | 2x    |     | Neg           |              |
| P012         |             | 2x    |     | M. spp        |              |
| P013         |             | 2x    |     | M. spp        |              |
| P014         |             | 2x    |     | M. spp        |              |
| P015         |             | 2x    |     | M. spp        |              |
| P012-<br>TTW |             |       |     | Neg           |              |
| P013-<br>TTW |             |       |     | Neg           |              |
| P014-<br>TTW |             |       |     | Neg           |              |
| P015-<br>TTW |             |       |     | Neg           |              |
| "C" Feedlot  |             |       |     |               |              |
| Sample No    | Animal ID   | Nasal | TTW | Cultured - NS | Cultured-TTW |
| C001         | DOF 14      | 2x    |     | M. spp        |              |
| C002         | DOF 14      | 2x    |     | M. spp        |              |
| C003         | Sick pen    | 2x    |     | M. spp        |              |
| C004         | Sick pen    | 2x    |     | M. spp        |              |
| C005         | Sick pen    | 2x    |     | M. spp        |              |
| C006         | DOF 140-150 | 2x    |     | M. spp        |              |
| C007         | DOF 140-150 | 2x    |     | M. spp        |              |
| C008         | DOF 140-150 | 2x    |     | Neg           |              |
| C009         | DOF 140-150 | 2x    |     | Neg           |              |
| C010         | DOF 140-150 | 2x    |     | M. spp        |              |

## Appendix E: Sampling flow charts and results for NP-BAL study.

Flow chart of 14 DOF sampling and results



Flow chart of 90 DOF sampling and results



## Appendix F: Summary of individual animal NP and BAL culture results.

First collection - December 2007

| (Sample No | Sample ID | Nasal | BAL | Cultured - NS       | Cultured-BAL        |
|------------|-----------|-------|-----|---------------------|---------------------|
| N-001      | 16 Swab   | 1x    |     | Mycoplasma isolated |                     |
| N-002      | 46 Swab   | 1x    |     | Mycoplasma isolated |                     |
| N-003      | 58 Swab   | 1x    |     | Mycoplasma isolated |                     |
| N-004      | 05 Swab   | 1x    |     | Few                 |                     |
| N-005      | 19 Swab   | 1x    |     | No                  |                     |
| N-006      | 27 Swab   | 1x    |     | Mycoplasma isolated |                     |
| N-007      | 37 Swab   | 1x    |     | No                  |                     |
| N-008      | 54 Swab   | 1x    |     | Mycoplasma isolated |                     |
| N-009      | 40 Swab   | 1x    |     | Mycoplasma isolated |                     |
| N-010      | 47 Swab   | 1x    |     | No                  |                     |
| N-001      |           |       | 1x  |                     | No                  |
| N-002      |           |       | 1x  |                     | Few                 |
| N-003      |           |       | 1x  |                     | Mycoplasma isolated |
| N-004      |           |       | 1x  |                     | Mycoplasma isolated |
| N-005      |           |       | 1x  |                     | No                  |
| N-006      |           |       | 1x  |                     | Mycoplasma isolated |
| N-007      |           |       | 1x  |                     | Mycoplasma isolated |
| N-008      |           |       | 1x  |                     | No                  |
| N-009      |           |       | 1x  |                     | Mycoplasma isolated |
| N-010      |           |       | 1x  |                     | No                  |
| Swab # 64  |           | 1x    |     | No                  |                     |
| Swab # 61  |           | 1x    |     | Mycoplasma isolated |                     |
| Swab # 62  |           | 1x    |     | Mycoplasma isolated |                     |
| Swab #88   |           | 1x    |     | Mycoplasma isolated |                     |
| Swab #86   |           | 1x    |     | Mycoplasma isolated |                     |
| BAL #64    |           |       | 1x  |                     | No                  |
| BAL #61    |           |       | 1x  |                     | Few                 |
| BAL #62    |           |       | 1x  |                     | No                  |
| BAL #88    |           |       | 1x  |                     | No                  |

|           |  |    |                     |                     |
|-----------|--|----|---------------------|---------------------|
| BAL #86   |  | 1x |                     | No                  |
| Swab #114 |  |    | Mycoplasma isolated |                     |
| Swab #102 |  |    | Mycoplasma isolated |                     |
| Swab #111 |  |    | Mycoplasma isolated |                     |
| Swab #130 |  |    | Mycoplasma isolated |                     |
| Swab #186 |  |    | Mycoplasma isolated |                     |
| Swab #190 |  |    | Mycoplasma isolated |                     |
| Swab #152 |  |    | Mycoplasma isolated |                     |
| Swab #201 |  |    | Mycoplasma isolated |                     |
| Swab #217 |  |    | Mycoplasma isolated |                     |
| Swab #173 |  |    | Few                 |                     |
| Swab #120 |  |    | Mycoplasma isolated |                     |
| Swab #172 |  |    | Mycoplasma isolated |                     |
| Swab #211 |  |    | Mycoplasma isolated |                     |
| Swab #144 |  |    | Mycoplasma isolated |                     |
| Swab #205 |  |    | Mycoplasma isolated |                     |
| Swab #226 |  |    | Mycoplasma isolated |                     |
| Swab #177 |  |    | Few                 |                     |
| Swab #200 |  |    | Few                 |                     |
| Swab #101 |  |    | Mycoplasma isolated |                     |
| BAL#217   |  |    |                     | Few                 |
| BAL#144   |  |    |                     | Mycoplasma isolated |
| BAL#130   |  |    |                     | Mycoplasma isolated |
| BAL#152   |  |    |                     | Mycoplasma isolated |
| BAL#111   |  |    |                     | Mycoplasma isolated |
| BAL226    |  |    |                     | Mycoplasma isolated |
| BAL#101   |  |    |                     | Mycoplasma isolated |
| BAL#200   |  |    |                     | No                  |
| BAL#120   |  |    |                     | No                  |
| BAL#133   |  |    |                     | No                  |
| BAL#154   |  |    |                     | No                  |
| BAL#211   |  |    |                     | No                  |
| BAL#201   |  |    |                     | No                  |

|                                       |  |  |                     |    |
|---------------------------------------|--|--|---------------------|----|
| BAL#173                               |  |  |                     | No |
| BAL#114                               |  |  |                     | No |
| BAL#172                               |  |  |                     | No |
| BAL#205                               |  |  |                     | No |
| BAL#186                               |  |  |                     | No |
| BAL#102                               |  |  |                     | No |
| BAL#177                               |  |  |                     | No |
| BAL#190                               |  |  |                     | No |
| BAL#133                               |  |  |                     | No |
| BAL#154                               |  |  |                     | No |
| Second collection date - January 2008 |  |  |                     |    |
| Swab #88                              |  |  | Mycoplasma isolated |    |
| Swab #111                             |  |  | Mycoplasma isolated |    |
| Swab #133                             |  |  | Mycoplasma isolated |    |
| Swab#114                              |  |  | Few                 |    |
| Swab#62                               |  |  | Few                 |    |
| Swab #226                             |  |  | Mycoplasma isolated |    |
| Swab #177                             |  |  | Mycoplasma isolated |    |
| Swab #46                              |  |  | Mycoplasma isolated |    |
| Swab #58                              |  |  | Mycoplasma isolated |    |
| Swab #61                              |  |  | Mycoplasma isolated |    |
| Swab #172                             |  |  | Mycoplasma isolated |    |
| Swab #154                             |  |  | Mycoplasma isolated |    |
| Swab #130                             |  |  | Mycoplasma isolated |    |
| Swab #27                              |  |  | Mycoplasma isolated |    |
| Swab #16                              |  |  | Mycoplasma isolated |    |
| Swab #40                              |  |  | Mycoplasma isolated |    |
| Swab #200                             |  |  | Mycoplasma isolated |    |
| Swab #37                              |  |  | Mycoplasma isolated |    |
| Swab #102                             |  |  | Mycoplasma isolated |    |
| Swab #120                             |  |  | Few                 |    |
| Swab #190                             |  |  | Mycoplasma isolated |    |
| Swab #47                              |  |  | Mycoplasma isolated |    |

|           |  |  |  |                     |                     |
|-----------|--|--|--|---------------------|---------------------|
| Swab #86  |  |  |  | Mycoplasma isolated |                     |
| Swab #64  |  |  |  | Mycoplasma isolated |                     |
| Swab #186 |  |  |  | Mycoplasma isolated |                     |
| Swab #205 |  |  |  | Mycoplasma isolated |                     |
| Swab #152 |  |  |  | Mycoplasma isolated |                     |
| Swab #217 |  |  |  | Mycoplasma isolated |                     |
| Swab #201 |  |  |  | Mycoplasma isolated |                     |
| Swab #114 |  |  |  | Mycoplasma isolated |                     |
| Swab #5   |  |  |  | Mycoplasma isolated |                     |
| Swab #19  |  |  |  | Mycoplasma isolated |                     |
| Swab #101 |  |  |  | Mycoplasma isolated |                     |
| Swab #173 |  |  |  | Mycoplasma isolated |                     |
| Swab #4   |  |  |  | Mycoplasma isolated |                     |
| BAL #111  |  |  |  |                     | Mycoplasma isolated |
| BAL #133  |  |  |  |                     | Mycoplasma isolated |
| BAL #101  |  |  |  |                     | Mycoplasma isolated |
| BAL #173  |  |  |  |                     | Mycoplasma isolated |
| BAL #172  |  |  |  |                     | Mycoplasma isolated |
| BAL #154  |  |  |  |                     | Mycoplasma isolated |
| BAL #27   |  |  |  |                     | Mycoplasma isolated |
| BAL #40   |  |  |  |                     | Mycoplasma isolated |
| BAL #200  |  |  |  |                     | Mycoplasma isolated |
| BAL #102  |  |  |  |                     | Mycoplasma isolated |
| BAL #120  |  |  |  |                     | Mycoplasma isolated |
| BAL #86   |  |  |  |                     | Mycoplasma isolated |
| BAL #205  |  |  |  |                     | Mycoplasma isolated |
| BAL #152  |  |  |  |                     | Mycoplasma isolated |
| BAL #217  |  |  |  |                     | Mycoplasma isolated |
| BAL #201  |  |  |  |                     | Mycoplasma isolated |
| BAL #114  |  |  |  |                     | Mycoplasma isolated |
| BAL #62   |  |  |  |                     | Mycoplasma isolated |
| BAL #226  |  |  |  |                     | Mycoplasma isolated |
| BAL #5    |  |  |  |                     | Mycoplasma isolated |

|          |  |  |  |  |                     |
|----------|--|--|--|--|---------------------|
| BAL #19  |  |  |  |  | Mycoplasma isolated |
| BAL #88  |  |  |  |  | No                  |
| BAL #130 |  |  |  |  | No                  |
| BAL #16  |  |  |  |  | No                  |
| BAL #37  |  |  |  |  | No                  |
| BAL #47  |  |  |  |  | No                  |
| BAL #177 |  |  |  |  | No                  |
| BAL #190 |  |  |  |  | No                  |
| BAL #4   |  |  |  |  | No                  |
| BAL #46  |  |  |  |  | No                  |

## **Appendix G: Documentation provided to practitioners along with the "necropsy kits" for the Necropsy Study.**

### **Project Objectives:**

Determine whether there are strain differences in the *Mycoplasma bovis* isolates from the nasal passages, infected lung tissue, and septic joint(s) of calves dying of presumptive *M. bovis* infections.

Determine whether there are 'lethal' strains of *M. bovis* that are common across multiple feedyards.

### **Case Selection:**

The target population is animals that have died or been euthanized as a result of *M. bovis* pneumonia or chronic pneumonia and polyarthritis syndrome (CPPS). We are only interested in calves that have died from *M. bovis*. The case definition for these calves is:

On gross post-mortem examination the calves lungs display pathognomonic lesions of *M. bovis*. These are randomly distributed raised micro-abscesses with caseous centers. (Pictures of typical *M. bovis* lungs have been included on the DVD disc included in this package.)

Animals displaying signs of CPPS. These animals must have lung pathology consistent with *M. bovis* and evidence of a septic arthritis. Calves that are lame but have little or no evidence of acute pneumonic process should not be recruited onto the study.

### **Samples Required:**

All animals selected for the study will require the following samples:

#### **Nasal Swab**

Nasal swabs should only be collected from animals that you euthanize; we are concerned that the nasal microflora changes in postmortem period and hence we are not interested in animals that are found dead (i.e. > 1 hr).

Ideally samples should be taken prior to euthanasia to avoid blood contamination, however, a bloody sample is better than no sample.

See the video and Powerpoint on the DVD provided for swabbing instruction.

Use the long swab provided. Write the calf number and date on the transport media vial. Freeze the samples, not refrigerate.

#### Swab of one arthritic joint/swollen joint

These may be collected through opening the joint as cleanly as possible. Typically the stifles and hock joints are affected. Only one affected joint requires swabbing.

Use the short swab provided. Write the calf number and date on the media container. Place in Freezer.

#### Lung tissue samples ~ 3cm by 3 cm minimum size:

Two samples of lung tissue acquired from the Diaphragmatic (also called the Accessory) Lung Lobe. Lesions must be included in the sample.

Place in purple top container provided.

Write the calf number and date on the container.

Place in Freezer.

#### Labeling:

The swabs and tissue containers must be labeled with a unique identification code and date of sampling. We suggest you use a simple alphanumeric code consisting of three logical letters that identify the feedyard followed by sequential numbering system, starting at "001". For example, Murray's feedyard might be identified as "MUR-001".

#### Sample Storage and Packaging:

- Place the 2 swabs and container with lung samples into the large zip lock bag provided. This is simply to keep the samples individually grouped.
- Complete the individual calf information sheet and place inside the zip lock bag with the samples.
- All samples should remain in the freezer until the time of shipping. Samples can be stored and shipped as a batch at convenient intervals. Samples should be sent by courier on ice; we will pay all the shipping costs. Prior to shipping please email either Nathan Erickson at [nathanerickson@canada.com](mailto:nathanerickson@canada.com) or myself at [murray.jelinski@usask.ca](mailto:murray.jelinski@usask.ca).

|                                                          |                                               |
|----------------------------------------------------------|-----------------------------------------------|
| Feedlot Name:                                            |                                               |
| Calf Tag #:                                              | Sample ID (XXX-###):                          |
| Date of Sampling (d/m/y)                                 |                                               |
| Date of Death (if different from date of sampling):      |                                               |
| Sex:     Steer                      Heifer               | Approximate # Days On Feed:                   |
| Gross PM signs consistent with <i>M. bovis</i> pneumonia | Yes<br>No (Pneumonia but perhaps not M bovis) |
| Signs of CPPS (pneumonia and arthritis)                  | Yes<br>No                                     |
| Additional comments:                                     |                                               |

# Appendix H. Summary of the "necropsy" study submissions.

| Veterinarian | Feedlot | Tag number | Date died collected | Samples    | Culture |                     | PCR      |
|--------------|---------|------------|---------------------|------------|---------|---------------------|----------|
|              |         |            |                     |            | Lung    | Joint               |          |
| J            | T       | 814-B4     | 12/13/2007          | Lung/swab  | 3+      | 1 colony            | M. bovis |
| J            | T       | 1302-B6    |                     | lung/joint | 3+      | positive subculture | M. bovis |
| J            | East    | 128-P509   | 12/27/2007          | Lung/swab  | 3+      | 1+                  | M. bovis |
| J            | East    | 29-P601    | 1/3/2008            | Lung       | 3+      | n/a                 | M. bovis |
| J            | East    | 125-P604   | 1/3/2008            | Lung/swab  | 3+      | 7 colonies          | M. bovis |
| J            | East    | 11-P801    | 11/2/2008           | Lung       | 3+      | n/a                 |          |
| J            | West    | 110-P804   | 12/31/2007          | Lung/swab  | 3+      | positive subculture | M. bovis |
| J            | West    | 183-P614   | 1/2/2008            | Lung/swab  | 3+      | positive subculture | M. bovis |
| J            | West    | 82-P707    | 12/14/2007          | Lung/swab  | 3+      | positive subculture | M. bovis |
| J            | West    | 184-P614   | 12/31/2007          | Lung/swab  | 3+      | 3+                  | M. bovis |
| J            | West    | 40-P810    | 1/2/2008            | Lung/swab  |         |                     |          |
| J            | West    | 20-P704    | 12/22/2007          | Lung/swab  | 3+      | Neg                 | M. bovis |
| J            | West    | 118-P614   | 12/31/2007          | Lung/swab  | 3+      | 3+                  | M. bovis |
| J            | West    | 80-P804    | 1/2/2008            | Lung/swab  | 3+      | 4 colonies          | M. bovis |

|      |          |          |            |                    |                        |                        |                                     |
|------|----------|----------|------------|--------------------|------------------------|------------------------|-------------------------------------|
| J    | West     | 32-P614  | 2/21/2008  | Lung/swab          | neg                    | n/a                    | M.bovis                             |
| J    | North    | 211-P304 | 12/29/2007 | Lung/swab          | 3+                     | 3+                     | Paenibacillus chibensis             |
| J    | North    | 169-504  | 12/28/2007 | Lung/swab          | 1+                     | positive<br>subculture | M. bovis/Paenibacillus<br>chibensis |
| J    | North    | 64-P506  | 1/3/2008   | Lung/swab          | 3+                     | positive<br>subculture | M. bovis                            |
| J    | North    | 77-402   | 6/12/2008  | Lung/swab          | Positive<br>subculture | 1+                     | M. bovis                            |
| J    | RPL      | 548-B3   | 1/20/2008  | Lung               | 4+                     | n/a                    | M.bovis                             |
| J    | RPL      | 1755-B9  | 1/14/2008  | Lung/swab          | 3+                     | positive<br>subculture | M. bovis                            |
| J    | Home     | 169-P116 |            | Lung               | 1+                     | n/a                    | M. bovis                            |
| Kent | BWF      | 2        |            | lung/joint/ns      | 2+                     | positive<br>subculture | M. ovipneumoniae/M. bovis           |
| Kent | Green    | 50       |            | lung/joint/ns      | 1+                     | Neg                    |                                     |
|      |          |          |            |                    |                        |                        |                                     |
| VAHS | Morrison | 72213#1  | 12/31/2007 | lung/joint<br>swab | 3+                     | Neg                    | M. bovis                            |
| VAHS | N        | 1311     |            | lung/joint<br>swab |                        |                        |                                     |
| VAHS | N        | 1037#2   | 6/3/2008   | lung/ns            | 3+                     | positive<br>subculture | M.alkalescens, P. chibensis         |

|      |    |          |            |                    |            |                        |          |
|------|----|----------|------------|--------------------|------------|------------------------|----------|
| VAHS | N  | 1292     | 12/21/2007 | joint swab         | n/a        | neg                    |          |
| VAHS | N  | 4715     | 3/6/2008   | Lung               |            |                        |          |
| VAHS | N  | 4623#5   | 3/6/2008   | lung/joint<br>swab | 2+         | positive<br>subculture | M. bovis |
| VAHS | N  | W4616#7  | 3/6/2008   | Lung               | 3+         |                        | M. bovis |
| VAHS | P  | 721/24   | 12/18/2007 | Lung               | neg        |                        | M. bovis |
| J    | T  | A3-539   | 12/9/2008  | lung/joint<br>swab | 4+         | 1+                     |          |
| J    | T  | A6-1317  | 12/16/2008 | Lung               | 4+         |                        |          |
| J    | T  | B3-571   | 12/16/2008 | lung/joint<br>swab | 1+         |                        |          |
| J    | T  | A6-1273  | 12/9/2008  | lung/joint<br>swab | 2 colonies | positive<br>subculture |          |
| J    | T  | B2-310   | 12/16/2008 | lung/joint<br>swab | 4+         |                        |          |
| J    | T  | A10-2625 | 12/16/2008 | lung/joint<br>swab | 4+         | 2+                     |          |
| J    | T  | A10-2592 | 12/16/2008 | lung/joint<br>swab | 4+         |                        |          |
| J    | T  | A10-2596 | 12/9/2008  | lung/joint<br>swab | 2+         | 4+                     |          |
| J    | LL | A11-2714 | 11/29/2008 | lung/joint         | 4+         |                        |          |

|   |      |          |            |                    |    |                        |  |
|---|------|----------|------------|--------------------|----|------------------------|--|
|   |      |          |            | swab               |    |                        |  |
| J | LL   | D12-1689 | 12/16/2008 | Lung               | 4+ |                        |  |
| J | LL   | B2-420   | 12/16/2008 | lung/joint<br>swab | 4+ |                        |  |
| J | LL   | D7-531   | 1/1/2009   | lung/joint<br>swab | 4+ | 1+                     |  |
| J | West | 404-84   | 1/7/2009   | lung/joint<br>swab | 4+ |                        |  |
| J | West | 303-226  | 1/3/2009   | lung/joint<br>swab | 2+ | neg                    |  |
| J | East | 310-88   | 12/7/2008  | lung/joint<br>swab | 4+ | positive<br>subculture |  |
| J | East | 311-60   | 12/7/2008  | Lung               | 4+ |                        |  |
| J | East | 210-24   | 12/8/2008  | lung/joint<br>swab | 1+ | 1+                     |  |
| J | East | 301-61   | 12/8/2008  | lung/joint<br>swab | 4+ | 1+                     |  |
| J | East | 311-53   | 12/8/2008  | lung/joint<br>swab | 2+ | 2 colonies             |  |
| J | East | 301-132  | 12/5/2008  | lung/joint<br>swab | 3+ |                        |  |
| J | East | 410-62   | 12/8/2008  | lung/joint         | 4+ | 1+                     |  |

|          |          |          |           |                    |                        |                        |          |
|----------|----------|----------|-----------|--------------------|------------------------|------------------------|----------|
|          |          |          |           | swab               |                        |                        |          |
| J        | East     | 209-161  | 12/7/2008 | lung/joint<br>swab | 4+                     | 4+                     |          |
| J        | East     | 209-177  | 12/8/2008 | lung/joint<br>swab | neg                    | 1+                     |          |
|          |          |          |           |                    |                        |                        |          |
| Campbell | U of S#4 | U of S#4 |           |                    | 3+                     |                        | M. bovis |
| Schumann | L        | 142-289  |           |                    | Neg                    | positive<br>subculture |          |
| Schumann | L        | 142-210  |           |                    | positive<br>subculture | positive<br>subculture |          |
| Schumann | L        | 146-1681 |           |                    | May-09                 |                        |          |
| Schumann | L        | W143-24  |           |                    | 6 colonies             |                        |          |
| Schumann | L        | W146-38  |           |                    | 2+                     | 1+                     |          |
| Schumann | L        | W148-159 |           |                    | positive subculture    |                        |          |
| Schumann | L        | W149-351 |           |                    | 4+                     |                        |          |
| Schumann | L        | 000-000  |           |                    | 1+                     | neg                    |          |
| Campbell | L        | R07-157  |           |                    | 3+                     | 3+                     |          |

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